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DEVELOPMENT OF A SYSTEM OF GENETIC ANALYSES FOR
RHODOMICROBIUM VANNIELII

by

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A thesis presented for the degree of
Doctor of Philosophy

Department of Biological Sciences
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SUMMARY

Preliminary experiments, to find phage or bacteriocin activity which might be plasmid-encoded amongst available strains of the Rhodospirillaceae, were unsuccessful, and no phage were isolated from the natural environment. The technique for phototrophic plate growth of Rm. vannielii was found to be unsuitable for basic genetic experiments and a method using microaerophilic growth was developed. The latter was found to yield better results in both mutagenesis and conjugation experiments despite an increased incubation time relative to phototrophic growth (i.e. 14 instead of 3-5 days to obtain colonies). The growth rate under microaerophilic conditions was increased by adding more yeast extract to the media and workable colonies were obtained after 10 days.

NTG and UV-mutagenesis were successfully used to isolate a variety of mutant types including pigment mutants, motility mutants and temperature sensitive motility mutants.

The pigment mutants were classified into four groups according to colour and spectroscopic analysis. A number of them were selected for further analysis. PAGE showed that in general these mutants were very similar to pigment mutants of other purple non-sulphur bacteria.

Transposon mutagenesis was successfully carried out using pSUP2021 and pJB4JI. Tn5-insertion pigment mutants, were isolated and Southern hybridization demonstrated that the transposon was inserted at single non-specific sites on the chromosome. This was verified by analysis of revertants obtained using a 'light-lethal' selection technique on a photosynthetically incompetent mutant. An EcoRI fragment containing Tn5 plus flanking sequences was subsequently cloned from this mutant into pBR322.

Conjugation experiments showed that broad-host-range plasmids from incompatibility groups P, Q and W could be transferred to and maintained in Rm. vannielii.

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I am grateful to Julie McCloskey for being with me and sharing 'what was' during the first couple of years of this project.

Finally thanks to Beverly Cook and Nicole Freeman for typing this thesis.

PS Thanks Mum.

DECLARATION

I declare that this thesis has been composed by myself from the results of research conducted under the supervision of Dr G P C Salmond and Dr C S Dow and has not been used in any previous application for a degree. All sources of information have been acknowledged by means of a reference.

Robbie Breadon

ABBREVIATIONS

A	-	absorbance
Ap ^r	-	Ampicillin resistant
Ap ^s	-	Ampicillin sensitive
ATP	-	adenosine 5'-triphosphate
bc ₁	-	cytochrome b-c ₁ oxidoreductase complex
Bchl	-	bacteriochlorophyll
Bphea	-	bacteriopheophytin
BSA	-	bovine serum albumen
cAMP	-	3',5' - cyclic adenosine monophosphate
cfu	-	colony forming unit
cpm	-	counts per minute
cs	-	cold sensitive
cyt	-	cytochrome
D	-	dalton
dATP	-	deoxyadenosine 5'-triphosphate
dCTP	-	deoxycytidine 5'-triphosphate
dGTP	-	deoxyguanosine 5'-triphosphate
dTTP	-	deoxythymidine 5'-triphosphate
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediamine tetra-acetic acid
em	-	electron microscope
g	-	gram
GDP	-	guanosine 5'-diphosphate
GS	-	glutamine synthetase
GTA	-	gene transfer agent of <u>Rhodobacter capsulatus</u>
GTP	-	guanosine 5'-triphosphate
hr	-	hour

ICM	-	intracytoplasmic membrane
Inc	-	plasmid incompatibility group
IR	-	infra-red
IS	-	insertion sequence
Kb	-	kilobase pairs
KD	-	kilodalton
L	-	litre
LHI	-	light harvesting complex I (primary)
LHII	-	light harvesting complex II (accessory)
M	-	molar
m	-	metre
MBHA	-	myxobacterial haemagglutinin
mg	-	milligram
μ g	-	microgram
MIC	-	minimum-inhibitory-concentration
min	-	minute
Mr	-	molecular ratio
mRNA	-	messenger RNA
NA	-	nutrient agar
NB	-	nutrient broth
NADH	-	reduced form of nicotinamide adenine dinucleotide
NADPH	-	reduced form of nicotinamide adenine dinucleotide phosphate
nm	-	nanometre
NTG	-	N-methyl-N'-nitrosoguanidine
OD	-	optical density
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate-buffered saline
PEG	-	polyethylene glycol
PM	-	pyruvate-malate minimal salts medium

PMY	-	PM medium supplemented with yeast extract
P + S	-	pairs and swarmer strain of <u>Rm. vannielii</u>
PSI	-	pounds per square inch
Q	-	quinone
RC	-	photosynthetic reaction centre
RNA	-	ribonucleic acid
SASP	-	small acid-soluble proteins
SDS	-	sodium dodecyl sulphate
sec	-	second
TEMED	-	N,N,N',N'-tetramethylethylenediamine
Tn	-	transposon
Tris	-	tris (hydroxymethyl) methylamine
ts	-	temperature sensitive
UV	-	ultra-violet light
v/v	-	concentration volume to volume
w/v	-	concentration weight to volume
YE	-	yeast extract

Abbreviations for antibiotics are listed in Table 2.5

CHAPTER 1

GENERAL INTRODUCTION

1.1 Prokaryotic systems used to study development

Due to their experimental potential and the ease with which they can be manipulated by genetic and biochemical techniques, bacteria have advantages over eukaryotes as systems in which to study development. Thus some of the most fundamental aspects of developmental biology can be studied more easily in bacteria than eukaryotes. This discussion will be restricted to bacteria that exhibit gross morphological changes as part of their normal life cycle, and that are amenable to genetic analysis, ie Bacillus, Myxococcus, Caulobacter and Streptomyces. Anabaena is also included because it is the first bacterial system in which DNA rearrangements have been correlated with morphological and biochemical changes at the whole-cell level.

1.1.1 Bacillus

The Bacillus life cycle

When encountering growth-limiting conditions, Bacillus spp. shift from vegetative growth to sporulation. Differentiation involves an asymmetric cell division with one daughter cell becoming a spore and persisting, and the mother representing a "dead-end" cell which eventually dies and lyses. The spore is metabolically dormant, optically refractile, and resistant to heat, UV light, lysozyme and a variety of chemical agents. Under suitable conditions the spore germinates, becoming once again a vegetative cell and thus completing a very simple life cycle. A great deal of research has gone into the sporulation part of the cycle so that the understanding of this process in Bacillus subtilis is perhaps the best characterized system of bacterial differentiation. The process of germination and outgrowth has only recently been given more attention,

although it is intrinsically linked to the process of endospore formation and development.

Morphological and biochemical changes occurring during sporulation

Once initiated, sporulation proceeds through six or seven arbitrarily-defined stages. Figure 1.1.1a shows the sequence of morphological events. It is during stages V and VI that the spores acquire, in succession, resistance to a variety of organic solvents and then to heat and to lysozyme; it is during this period that their characteristic germination properties appear. All the properties develop in parallel with the laying down of coat proteins. The latter process involves a self-assembly of proteins that have been synthesized previously. Thus sporulation can be divided into two qualitatively distinct phases (Mandelstam and Errington, 1987). In the first there is a modified (ie asymmetric) cell division during which gene expression occurs and results in the production of a vegetative pre-spore with its own cell wall. In the second phase it is transformed to a mature spore using preformed coat proteins.

Induction or initiation of sporulation

Very little is known about how B. subtilis senses nutritional stress or how its response to stress is translated into activation of specific genes. However, it is clear that the stage of DNA replication is important and that guanine nucleotides (GDP and GTP) and other highly phosphorylated nucleotides may be involved. For discussion of possible mechanisms see Dworkin (1985) and Losick et al (1986).

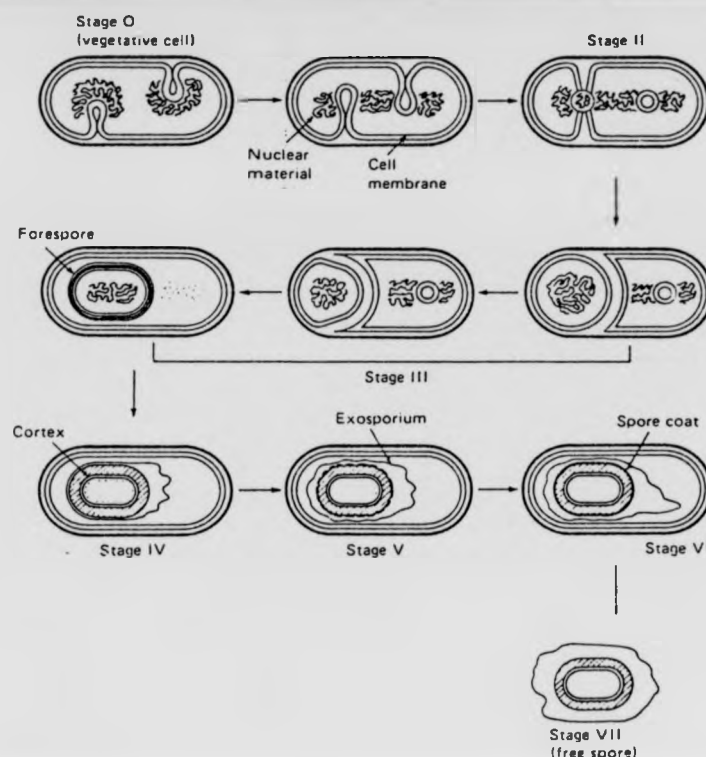


Figure 1.1.1a The sequence of morphological events during sporulation in Bacillus

Stages

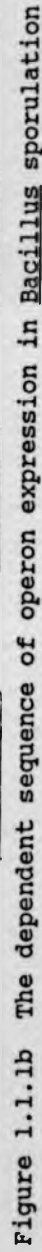
- 0 The vegetative cell
- I Pre-septation : chromosomes fuse to form an axial filament;
- II Septation : chromosomal separation followed by asymmetric cell division;
- II Engulfment : the mother cell septum engulfs the 'forespore' enclosing it in a second membrane of reversed polarity;
- IV Cortex formation : cell wall peptidoglycan is laid down between the double membrane of the spore protoplast;
- V-VI Coat formation and maturation : polypeptides are laid down in concentric layers to form spore coat and the spore shrinks;
- VII Release : the completely mature spore is released by lysis of the mother cell.

(adapted from Dworkin, 1985).

Regulation of sporulation

A number of lines of evidence lead to the conclusion that the orderly appearance of events associated with sporulation are, to some extent, a result of the regulated expression of genes that control the event. The data include the sequential and periodic synthesis of mRNA and proteins, analysis of mutants, work with lacZ fusions and RNA-DNA hybridization techniques using cloned genes. The latest model (Mandelstam and Errington, 1987) is shown in Figure 1.1.1b. A dependent sequence branches at an early stage so that there are independent lines of expression in the mother cell and the spore. Further branches occur in both cell compartments.

Evidence for transcription of sporulation genes being regulated during differentiation is shown by changes in RNA polymerase accessory factors (in B. subtilis there are at least nine species of sigma factor) and production of sporulation-specific mRNA species. For further discussion of this aspect of the regulation of sporulation see Losick and Pero (1981) and Losick and Youngman (1984). Analysis of the sequences of cloned regulatory genes suggest that other types of regulation (eg DNA-binding proteins and proteolytic enzymes) may play a greater role in sporulation than previously envisaged (Mandelstam and Errington, 1987). Evidence for the involvement of translational control in sporulation comes from the isolation and analysis of antibiotic-resistant mutants and work with in vitro translation systems prepared from B. subtilis (Dworkin, 1985). In contrast, analysis of a series of sporulation genes showed that there was no delay between the appearance of mRNA's and production of their corresponding proteins (Mandelstam and Errington, 1987).



Genetic control of spore development is exerted by about 50 known operons, some of them poly-cistronic,

scattered around the chromosome. They are expressed in a branched dependent sequence which is largely completed in the first four hours. Regulatory genes so far identified include: Sigma factors (OH, IIA, IIGB, IID, IIIG);

DNA binding proteins (IID, IIC, gerE); and proteolytic enzymes.

The regulation of gene expression observed during sporulation cannot be accounted for by the accessory sigma factors of RNA polymerase alone. Other processes such as post-translational processing and self-assembly into higher order structures may also be involved. The most recent research shows that the morphological changes observed are the result of a branching cascade of interactions (Mandelstam and Errington, 1987).

1.1.2 Caulobacter

The life cycle of *Caulobacter crescentus*

In Caulobacter the events of developmental interest are embedded in, and coordinated with, the growth of the cell rather than being alternative to it. This is somewhat similar to the situation found in Rhodomicrobium which will be discussed later. Figure 1.1.2a illustrates the C. crescentus life cycle. There are two coexisting, functionally-distinct types of cell in the population - the swarmer cell and the stalked cell. The motile swarmer cell carries a single polar flagellum, DNA-phage receptors and pili at one pole. The swarmer cell differentiates into a sessile stalked cell by simultaneously shedding the intact flagellum, the pili and phage receptors and growing a stalk at the same site. The stalked cell elongates and upon completion of a round of DNA replication, a new flagellum, pili and phage receptor sites are formed at the stalk-distal pole of the incipient swarmer cell. Cell division yields two different daughter cells: a stalked cell and a flagellated swarmer cell. The progeny stalked cell continues to function as a stem cell giving rise to a new swarmer cell at each division. Chromosome replication is blocked in the progeny swarmer cell but not in the progeny stalked cell.

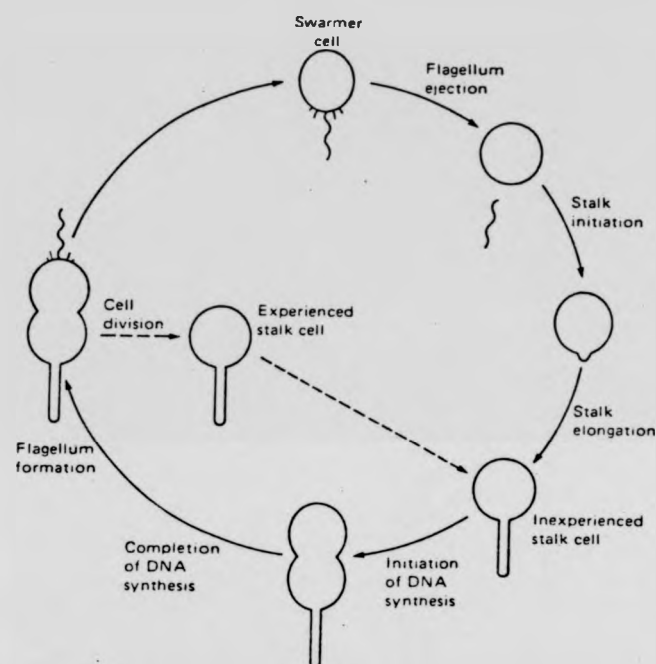


Figure 1.1.2a The life cycle of C. crescentus
(from Dworkin, 1985)

Temporal patterning of gene expression in *C. crescentus*

As described above, various activities of *Caulobacter* are oriented in time. Pili, holdfasts, flagella and stalks, in addition to all the normal growth and divisional structures such as septa, are synthesized only at specific times. DNA synthesis is also temporally regulated and DNA replication acts as a cellular clock that controls the timing of other events. The regulation of the synthesis and assembly of the *Caulobacter* flagellum has been intensively studied to the level of gene expression. This work has been extensively reviewed (Dworkin, 1985; Shapiro, 1985; Newton, 1987). The flagellum is composed of a basal body-rod assembly, a hook and a filament. The structure and its polypeptide composition are illustrated in Figure 1.1.2b. About 30 *fla* genes are required for flagellum formation and most of these are located in one of three clusters (Figure 1.1.2c) representing the basal body genes, the hook genes and the flagellin structural genes, respectively. Genes from each of these major clusters have been isolated. They are organized in the regulatory hierarchy shown in Figure 1.1.2c. In the *fla* EY gene cluster the expression of each gene is regulated by the genes above it and the genes are positively regulated by a cascade of trans-acting factors. Flagellin gene expression is programmed in an ordered sequence (29-, 27- and 25-kD flagellin gene products). Thus the genes are transcribed in the order of assembly of their products into the flagellum. Sequential gene expression is also observed in the hook cluster. The trans-acting regulatory cascade proposed by Newton (1987) (shown in Figure 1.1.2c) could determine the order, if not the time, of *fla* gene expression. The 5' regulatory regions of certain *fla* genes have conserved sequences (at nucleotides -100 -24 and -13), and their

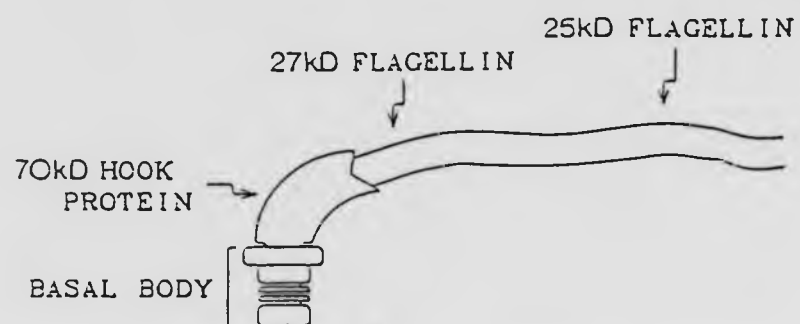


Figure 1.1.2b The *C. crescentus* flagellum
(from Newton, 1987)

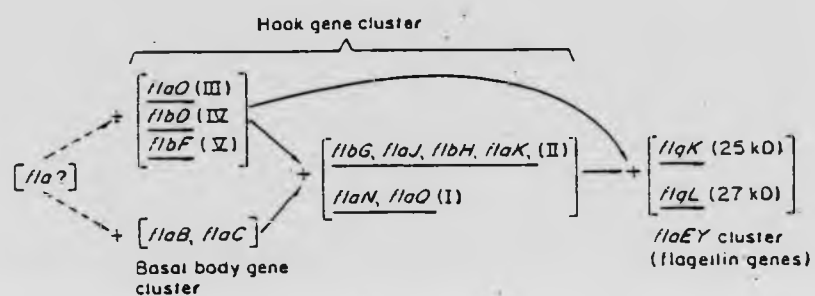


Figure 1.1.2c Hierarchical organization of fla genes in C.crescentus

The three major gene clusters mapped on the chromosome are depicted, but not all genes in each cluster are included.

(from Newton, 1987)

presence has been correlated with a common pattern of regulation. These consensus sequences are likely to play a role in the transcription and differential expression of fla genes in the C. crescentus cell cycle (Newton, 1987).

Spatial localization of gene products

Differentiation in C. crescentus requires that the temporal pattern of gene expression discussed above be closely coordinated with the spatial localization of the assembling gene products. It has been shown that the polar portion of the cell membrane contains a unique set of proteins not found in the remainder of the cell membrane. Newly-synthesized proteins including flagellin are compartmentalized within the flagellated portion of the pre-divisional cell. Flagellin monomers are apparently targetted to the cell pole for assembly at an early stage of translocation to the membrane. Thus the C. crescentus cell envelope is both physically and functionally differentiated into at least two relatively stable domains - the flagellated cell pole and the remainder of the cell envelope.

Shapiro (1983) has suggested that the temporal and spatial aspects of development in C. crescentus may be jointly controlled by nucleoid-membrane interactions determining the site of assembly of new organelles and the timing of their synthesis by DNA replication.

1.1.3 Anabaena : Heterocyst development and spacing

Anabaena cylindrica normally grows in long filaments composed of photoautotrophic vegetative cells. If fixed nitrogen becomes limiting, heterocysts, which have the ability to fix dinitrogen, develop.

Heterocysts are an answer to the dilemma of nitrogen fixation (using an extremely O_2 -sensitive enzyme) in an O_2 -evolving photoautotroph. In the heterocyst the O_2 -generating-photosystem II is switched off and the cell is surrounded by an oxygen-impermeable layer. Photosynthesis occurs, as usual, in the neighbouring vegetative cells which supply the heterocysts with the metabolites they lack as a result of restricted photometabolism. Heterocysts are formed along the filament at regularly spaced intervals, initially as non-committed precursor cells called proheterocysts (4-5 hrs) and later becoming irreversibly mature heterocysts (14-32 hrs). The spacing of heterocysts is in part dictated by asymmetric cell division and by a zone of inhibition on either side of the heterocyst. The inhibition is generated by a diffusible inhibitor (possibly glutamine) excreted by proheterocysts.

Glutamine synthetase (GS) is required for N-metabolism in both vegetative cells and heterocysts. In these two differentiated cells the same GS gene is transcribed from different promoters. For a more detailed discussion of the physiology, biochemistry and development of heterocysts the reader is referred to Wolk (1982).

Nitrogenase genes and DNA rearrangements

Cloning and physical mapping of *A. cylindrica* nitrogenase genes from vegetative cells showed that their organization differed from that of the well characterized *nif* genes of *Klebsiella pneumoniae* (Rice *et al.*, 1982). In particular, the *nifHD* and *nifK* genes of *Anabaena* are separated by 11kb of DNA, whereas these genes are adjacent and cotranscribed in *Klebsiella*. This 11kb of DNA is excised from the chromosome during heterocyst differentiation and is found as a circle in the mature heterocyst (Golden *et al.*, 1985). The rearrangement permits the entire *nifHDK* operon to be transcribed from the *nifH* gene promoter. This,

together with another rearrangement, occurs at approximately the same late stage of heterocyst development, after morphological changes are readily apparent (Haselkorn *et al.*, 1986; Golden *et al.*, 1987). Thus these DNA rearrangements are a consequence of differentiation, not a triggering event.

1.1.4 Streptomyces

The Streptomyces genus (Streptomyces coelicolor and Streptomyces lividans) has been the focus of most developmental biology of the actinomycetes (Chater, 1984) and has a well developed system of genetic analysis (Hopwood *et al.*, 1986). Streptomyces exhibit a complex cycle of morphological development (Figure 1.1.4a); they form a branching, substrate mycelium that is occasionally interrupted by a crosswall. When faced with nutrient depletion the population begins to form aerial mycelium as branches of the substrate mycelium. At the ends of these aerial hyphae, the cells differentiate into a chain of spores. The population of substrate mycelium undergoes massive lysis. Thus the colony is an example of multicellular and spatial differentiation. The morphological changes that occur with the conversion of aerial mycelium to hyphal spores have been well characterized and are summarized in Figure 1.1.4b. The resultant spores are slightly more heat resistant than aerial mycelia, are resistant to desiccation and are metabolically dormant.

Regulation of developmental events

Developmental-specific genes have been identified by mutations affecting the production of aerial mycelium (bald-bld-mutations) or spores (white-

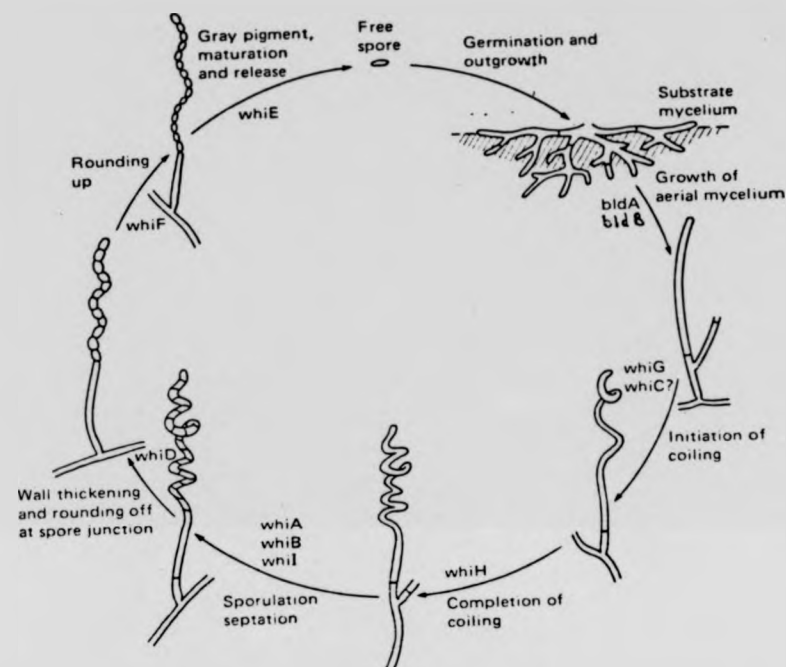


Figure 1.1.4a The developmental cycle of *S.coelicolor*

The genes thought to be involved at each stage are shown.

(Adapted from Dworkin, 1985)

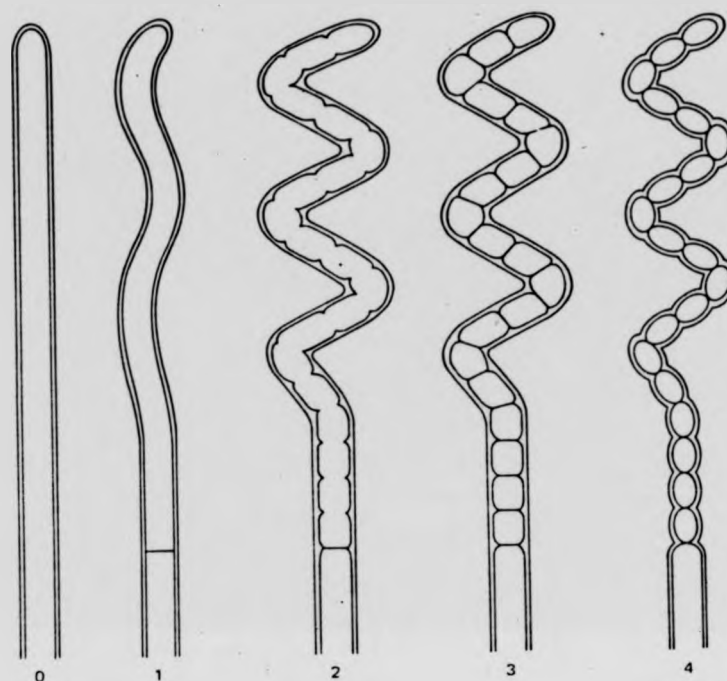


Figure 1.1.4b Morphological stages of sporulation in S. coelicolor

Stage

- | | |
|---|--|
| 0 | Vegetative growth |
| 1 | Sporulating hyphae are divided into long cells by ordinary crosswalls, and the tips begin to coil; |
| 2 | The apex is partitioned into spore-sized compartments by sporulation septa; |
| 3 | The cell walls thicken and constrictions appear between the young spores. |
| 4 | As the spores mature, they round off and separate. |
- (from Dworkin, 1985)

whi-mutations). Several of these genes have been cloned, including: those for the biosynthesis of A-factor, a diffusible compound needed for the initiation of sporulation; two genes needed in the very early stages of aerial mycelium development in S. coelicolor, bldA and bldB; and the whiG gene, the product of which is important in an early stage of the metamorphosis of aerial hyphae into spore chains. The promoter regions of genes cloned so far are unexpectedly complex and there is good evidence that multiple forms of RNA polymerase are involved in selecting a variety of classes of promoter sequence (Chater, 1984). bldA mutations are specifically located in a tRNA-like sequence and the tRNA-like molecule is specific for the rare leucine codon UUA. Use of this codon may allow translational control of development (Lawlor *et al.*, 1987). In whiG mutants of S. coelicolor aerial hyphae do not show any sign of sporulation and whiG is thought to be a key gene triggering the onset of sporulation in aerial hyphae. The whiG mutation is epistatic to representative whiA, whiB, whiH and whiI mutations and the presence of additional copies of whiG cause hypersporulation. It has been suggested that whiG is likely to be a sigma factor (Mendez and Chater, 1987).

1.1.5 The Myxobacteria

Myxobacterial life cycles

The Myxobacteria are Gram-negative, rod-shaped soil bacteria that have two unusual properties: gliding motility and their unique life cycle. In the growth cycle the rods grow and divide by conventional transverse binary fission until growth becomes limited. If three conditions are met (nutrient depletion, a solid surface and high density) the cells shift to an alternative cycle of development. The life cycle of Myxococcus xanthus is shown in Figure 1.1.5a. Development results in the formation

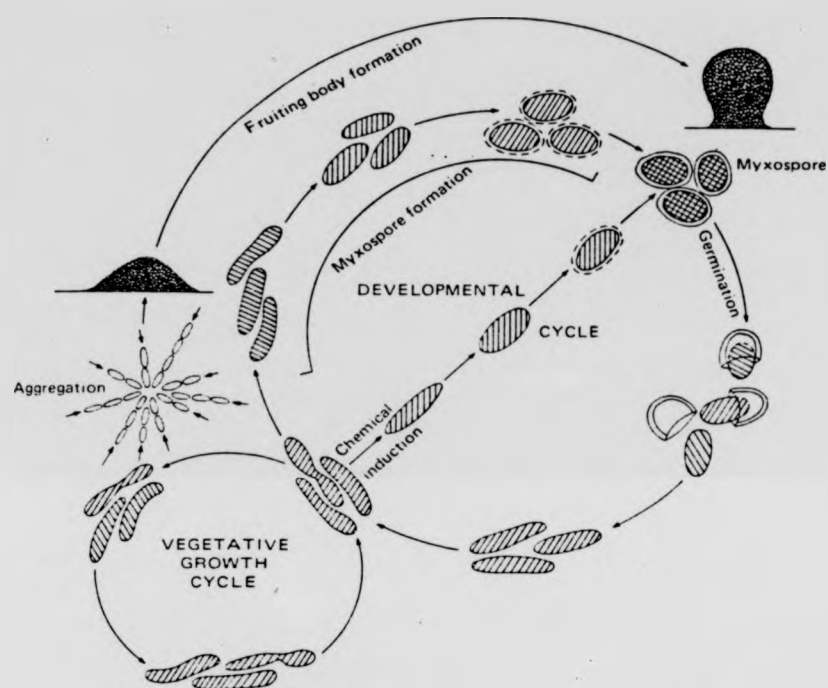


Figure 1.1.5a The life cycle of *M. xanthus*

(not drawn to scale) (from Dworkin, 1985)

of elevated mounds in M. xanthus and more elaborate structures in other species, eg Stigmatella aurantiaca. Thus they exhibit colonial morphogenesis involving cell interactions and communication and cellular morphogenesis in that resistant, metabolically-quiescent cells (myxospores) are found within the fruiting bodies. Fruiting bodies are stable until conditions induce myxospore germination whereupon the whole process of swarming, feeding and development takes place again. The process of cellular morphogenesis can be rapidly (ca. 90min) and synchronously induced in suspension cultures by the addition of 0.5M glycerol. The colonial development on appropriate agar medium takes up to 72 hrs to obtain mature fruiting bodies. Most of what is known about spore formation is derived from studies of glycerol induction. Myxobacteria perform two types of fascinating social behaviour: aggregation which occurs prior to fruiting body formation, and rippling in which the swarming cells form oscillating waves. Gliding motility is required for both types of behaviour. Several mechanisms have been suggested for gliding motility, but none of these have been proven. Development and behaviour in Myxobacteria have been recently reviewed (Reichenbach, 1986; Shimkets, 1987).

Control of development

Development can be divided into three stages based on study of mutants - initiation, aggregation and fruiting body formation and sporulation.

Initiation

In M. xanthus, amino acid starvation is the major environmental impetus for initiating development. The nature of the intracellular signals

involved is poorly understood. As cells enter development a number of changes occur in cellular RNA. Amongst a subclass of stable RNA is the message for protein S which is the major spore coat protein from fruiting body spores and its synthesis begins very early in cell conversion.

Aggregation

This process requires nutritional deprivation, a solid surface, high cell density and in some species, light. A large number of aggregation-deficient mutants have been isolated and many of them contain a motility or behavioural defect (Shimkets, 1987). These may include defects in cell interactions necessary to coordinate group movement, or difficulties in controlling the direction of cell movement. One protein which is involved in cell interaction and is produced during aggregation is a lectin known as MBHA.

Sporulation

The developmental pathway leading to sporulation involves cell communication, and results in the death of the majority of cells. Study of conditional nonsporulating mutants has suggested that at least two intercellular signals are involved. Results from temperature-shift experiments with ts-mutants, and expression of developmentally-regulated promoters in mutant backgrounds, suggest that the sporulation pathway begins very early in development, perhaps as early as aggregation. The mechanism of control of lysis of 70% of the population during development is little understood, but it is thought to be involved in the sporulation pathway rather than in aggregation since all known

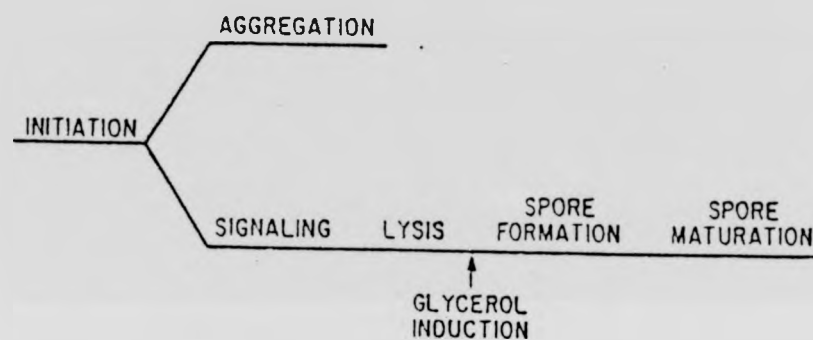


Figure 1.1.5b Organization of the developmental program of *M.xanthus*
(from Shimkets, 1987)

mutants in developmental autolysis also fail to sporulate. A defined series of physiological changes occurs in the sporulation branch of development. An initial period of cell-to-cell contact during which intercellular signalling occurs, is required. This is followed by a pre-divergence phase in which all cells are committed to develop but all have similar fates if washed and placed in fresh medium. In the third phase the cells are committed to their respective developmental fates (lysis or spore). Initially the spores are optically-refractile but not resistant. Resistance properties develop in a final maturation phase. A model for the developmental program (Shimkets, 1987) is shown in Figure 1.1.5b.

1.1.6 Summary

There is a diversity of prokaryotic developmental systems, each with peculiar experimental virtues and short-comings, and for each there are aspects of development that are either uniquely or more conveniently studied in that system (Table 1.1.6). One interesting aspect of differentiation that is common to the Bacillus, Caulobacter, Anabaena and Rhodospirillum rubrum systems is that of asymmetric cell division leading to the production of two cells with different developmental fates. In Bacillus, Caulobacter and Rhodospirillum rubrum the smaller or daughter cell acts as a progenitor or growth-precursor cell which is carried through to the next generation of the life cycle. In Bacillus and Rhodospirillum rubrum the next cycle begins when environmental conditions are suitable. The spores produced by Streptomyces and Myxobacteria under conditions of nutrient depletion perform similar resting-cell functions. In contrast, the heterocyst of Anabaena, although derived from the smaller cell of an asymmetric division is a 'dead-end' cell,

Organism	<u>Bacillus</u>	<u>Caulo-</u> <u>bacter</u>	<u>Anabaena</u>	<u>Strepto-</u> <u>myces</u>	<u>Myxococcus</u>	<u>Rhodo-</u> <u>microbium</u>
Well charact- ized system	✓	✓	✓	✓	✓	✓
Extensive changes in morphology	✓	✓	✓	✓	✓	✓
Cyclic process	✓	-	-	✓	✓	✓
Reversible change	✓	-	-	✓	✓	✓ exospore
Cell cycle independent	✓	-	✓	✓	✓	✓ exospore
Cell cycle dependent	-	✓	-	-	-	✓ swarmers
Genetics/ molecular biology well developed	✓	✓	-	✓	✓	-
Homogenous cell populations for synchrony	✓	✓	-	-	✓	✓
Motility/ chemotaxis	-	✓	-	-	✓ gliding	✓
Multicell- ularity	-	-	✓	✓	✓	✓
Cell-cell interactions/ signalling	✓	-	✓	✓	✓	?
Special features	mother- cell -spore inter- action through membrane system	temporal and spatial control of gene express- ion	DNA rearran- gement	DNA rearran- gement	social behaviour gliding motility surface inter- action	temporal & spatial control of gene express- ion includ- ing photo- system

Table 1.1.6 Comparison of the prokaryotic developmental systems

(✓ = present; - = not present)

its only function being to fix atmospheric nitrogen so that the rest of the population can survive. The lysis: of the mother cell in Bacillus: of 70% of the Myxococcus population during sporulation; and of the substrate mycelium during aerial sporulation in Streptomyces could similarly be considered examples of bacterial altruism.

Rhodomicrobium exhibits certain aspects of development that are found in the other systems and is most like the Caulobacter system. However it has some unique characteristics and experimental virtues which make its study worthwhile. These are discussed later. To understand and compare the regulatory phenomena that underlie the developmental events in the various systems a genetic/molecular approach is needed. The next section considers the genetic approach to studying development in prokaryotes.

1.2 Genetic techniques for studying development in prokaryotes

1.2.1 Classical genetics

There are a variety of classical genetic techniques that have been designed to analyse complex processes (eg development) in microorganisms. These were classed into four levels of genetic analysis by Botstein and Maurer (1982): mutant isolation and characterization; tests of epistasis; determination of order of gene function; and pseudoreversion analysis. Another good review of the classical approaches to the genetic analysis of differentiation in bacteria is that by Piggot (1979).

Mutant isolation and characterization

This is the first and indispensable step in any genetic analysis and involves definition of phenotypes that can be distinguished as being specific to the process. In the case of cell cycle-independent development (eg sporulation in *B. subtilis*) mutants can grow vegetatively but are unable to complete the alternative differentiation cycle. In the case of cell cycle-dependent development the mutants are often necessarily conditional-lethals that grow normally under permissive conditions but display distinctive phenotypes under restrictive conditions. A collection of mutants are sorted into loci by genetic mapping and into functional groups (genes/operons) by complementation analysis. Using a large number of independent mutants it is possible to determine the number of genes involved in the process and this is a useful indicator of its complexity. Complementation analysis requires a method of placing the mutant genes from two different mutants in the same cell. This can be accomplished either by using a temperate

phage to introduce the mutant gene or a stable plasmid to generate the partial diploid. If the mutations are in different genes, phenotypic complementation will allow the process to occur. If the mutations are in the same gene, complementation will not occur. Characterization of mutants can yield much information about the process under study (eg antibiotic-resistant mutants that are also developmentally-defective) and can reveal processes that are otherwise obscure (eg phenotypic complementation in M. xanthus has shown the involvement of cell-cell contact and intercellular signalling in motility and development). Such studies do not require a system of genetic exchange and the type of information that can be obtained is reviewed by Piggot (1979).

Tests of epistasis

These can be used to clarify the sequence of events in, and the structure of, a developmental pathway. The idea behind this kind of analysis is that a double mutant carrying two mutations which confer distinguishable phenotypes, and that affect different points in the same dependent pathway, will have a predictable phenotype; ie the phenotype of the double mutant is conferred by the mutation, the effect of which occurs earlier in the pathway. This method requires relatively little of the genetic system other than the ability to construct double mutants. However these tests require detailed knowledge of the mutant phenotypes and are complicated by a number of variables such as whether one (or both) gene(s) is a regulatory element. Nevertheless, it has been used successfully to determine the nature of some parts of the developmental processes of B. subtilis, C. crescentus and M. xanthus.

Direct tests of order of function

It is very common, in developmental or morphogenetic pathways, to find many genes with indistinguishable mutant phenotypes. In such cases, simple tests of epistasis cannot be carried out. An alternative approach is the reciprocal-shift method which requires the ability to use different methods to block the expression of each of the two genes whose order one is trying to determine. Using conditional mutants that are either heat-sensitive or cold-sensitive has thus far been the method of choice. The logic of the experiment is that if genes A and B are expressed in that order, in a linear dependent pathway, and if gene A is cold-sensitive whilst gene B is heat-sensitive, then applying a restrictive high temperature followed by a shift to a restrictive low temperature should allow the sequential expression of both A and B. Reversing the sequence of temperature shifts, or if the gene sequence is B, A, should result in no expression. Furthermore, if both sequences of temperature shifts (hot to cold and cold to hot) allow expression of the pathway, then the A and B mutations are on independent pathways. Finally, if A and B are on separate but interdependent branches of a pathway, then either shift should prevent expression of the pathway. These results are illustrated in Table 1.2.1. Thus the reciprocal-shift method is useful for determining not only the sequence of action of genes in a pathway but also provides information on the structure of the pathway.

Pseudoreversion methods

These can be used to detect and study assemblies of interesting gene products which often cannot be identified by biochemical or

Table 1.2.1 Results expected in reciprocal shift experiments using *cs* *ts* double mutants.

(From Botstein and Maurer, 1982)

Dependency relationship	Result of shift	
	17°—37°	37°—17°
Dependent <i>ts</i> → <i>cs</i> →	+	— ^a
Dependent <i>cs</i> → <i>ts</i> →	—	+
Independent <i>ts</i> → <i>cs</i> →	+	+
Interdependent (<i>cs</i> , <i>ts</i>)→	—	—

^aA "+" indicates passage to a second cell cycle (i.e., two arrested cells are found and a "—" indicates arrest in the first cell cycle (i.e., one arrested cell is found).

morphological examination. In cases where two molecules or subcellular assemblies interact with each other, certain types of suppressor mutations can be extremely valuable. In general, an extragenic suppressor mutation is one that occurs in a gene physically distant from another mutation but which acts to reverse the effect of the first mutation. Thus it appears as a reversion of the primary mutation, but is more properly referred to as a pseudoreversion. A particularly useful type of suppressor mutation is one referred to as an interaction suppressor. If the function of a particular gene product is to interact with another gene product, one consequence of a mutation will be to alter that gene product so that it can no longer interact properly. An interaction suppressor mutation would then result in a modification of the second reactant so that it can once again interact with the other mutated gene product. Suppressors are of relatively little interest in studies of development unless some way can be found to determine the normal functions of the suppressor gene. A simple solution to this problem is to screen among the revertants for mutations, that, in addition to the suppressor phenotype, have a new phenotype of their own. Thus a general scheme for mapping gene interactions in vivo emerges: interaction suppressors having an additional phenotype are obtained from a developmental mutant, and the new phenotype serves to identify the suppressor gene. Pseudorevertants selected for having new phenotypes are also useful both for finding new mutations in previously identified genes and for identifying additional genes involved in a particular process. This includes ones that are intimately involved in the developmental pathway but have other non-development, but essential, functions that would be missed in searches for mutants with developmental phenotypes. Proper use of pseudoreversion analysis entails the ability to carry out rather sophisticated genetic manipulations and

its use as a major genetic tool has been limited, especially in bacteria.

1.2.2 The 'new' genetics

Although the classical methods summarized above clearly have the power, in principle, to allow extensive genetic analysis of developmental pathways, their application has been limited by the difficulties of the genetic manipulations in bacteria. Many of the difficulties have been reduced by the 'new' genetic technology. It not only circumvents the difficulty in most organisms of moving genes from one cell to another but allows the selection of otherwise nonselectable properties (eg developmental processes) as well as the in vitro modification of isolated genes. The key elements are relatively few: plasmids, restriction endonucleases and ligases, temperate phage and transposons.

The discovery of transposable elements carrying genes conferring antibiotic resistance facilitated a large number of new methods of in vivo genetic manipulation in prokaryotes. Many of these have become standard and have been reviewed (Kleckner et al., 1977). The most useful transposons are pieces of DNA containing one or more genes encoding drug resistance and flanked by two insertion sequences. These insertion sequences can cause the transposon to be inserted by means of 'illegitimate recombination', at almost any site along the genome in the case of Tn₅. This has two main consequences. Firstly, the gene into which the transposon is inserted is interrupted and its function destroyed. Thus, a transposon is in effect a mutagen that generates polar, null mutations. Secondly, a selectable marker (drug resistance) is inserted as part of the transposon. Thus, any gene that can be cotransduced with

the transposon can be coselected. This is particularly useful in the isolation of non-selectable, eg developmental genes. All that is needed is a way to get a transposon into the cell (usually a phage or a plasmid) and a good generalized transducing phage. These are available in M. xanthus, Caulobacter and Bacillus. They have been fairly extensively employed in M. xanthus which can be infected by coliphage P1 carrying a Tn5 transposon. P1 injects its DNA into M. xanthus but neither replicates nor lysogenises. Thus it provides a perfect 'suicide' vector since cells become resistant to Kanamycin only when Tn5 has transposed to a chromosomal location. This system has been used to tag otherwise unselectable developmental genes in M. xanthus with the transposon-borne antibiotic marker (Kuner and Kaiser, 1981). The Streptococcus transposon Tn917 has been used in a similar fashion by insertion into B. subtilis via the phage SP β (Youngman *et al.* 1985). This has facilitated cloning of developmentally-regulated genes from Bacillus spp. in E. coli.

Another aspect of the use of transposons addresses the problem of expression of developmental genes. Often the expression of such genes is subtle, the gene products are unidentified, or the assay for gene expression is awkward or difficult. By fusing the promoterless structural gene for β -galactosidase (lacZ) to a transposon that contains a gene for antibiotic resistance (eg Kanamycin resistance in Tn5), one can insert the lacZ gene into the chromosome or plasmid of a developing cell. The virtue of this approach is that the availability of chromogenic substrates for β -galactosidase allows one to visualise, by a simple colour change in the colony, whether or not the gene is being expressed. Tn5-lacZ fusions have been used to study developmentally regulated genes in both C. crescentus (Newton, 1987) and M. xanthus and

Tn917 has been used similarly in B. subtilis (Youngman *et al.*, 1985).

For most organisms it is now straightforward to make recombinant DNA libraries that are virtually certain to contain every gene of the organism. The cloned DNA fragments can be very large with respect to a single gene or operon, and they can be generated in ways that approximate randomness. Therefore a complete library will contain a gene many times with varying amounts of flanking DNA, ensuring that at least sometimes the genes or operons are recovered with their flanking regulatory sites intact. Once the DNA has been cloned in E. coli it is possible to amplify the gene or gene products or to study the gene's expression in the context of an E. coli regulatory system. Subsequently, the gene may be analysed in great detail, to determine its product and nucleotide sequence.

In most microorganisms with developed genetics (eg B. subtilis, M. xanthus and C. crescentus) there are ways of returning the cloned genes in libraries to the organism by DNA transformation, phage infection or conjugation. Using mutations that show a defect, it is therefore usually possible to isolate any gene by its ability to complement the defect. By subcloning smaller and smaller fragments, it is also possible to identify structural genes with certainty, and to separate control regions from structural genes. With the ability to reinsert a fragment of DNA back into a cell from which it came, the various developmental consequences of complementation, gene dosage and localized mutagenesis can be examined. The DNA may be returned on plasmids which replicate freely in the organism under study. If no such vector is available then an integrational vector may be used. Integrational vectors have been used most successfully in M. xanthus and B. subtilis. Such vectors

cannot replicate but carry a piece of DNA homologous to a chromosomal locus. When this plasmid is introduced into the host, the plasmid can integrate into the chromosome through a single crossover in the region of homology, forming a structure that resembles a tandem duplication except for the foreign plasmid vector sequences located at the novel joint. Such merodiploids can also be constructed using transposon technology. Integrational vectors are very attractive alternatives to transposons for such manipulations as insertion mutagenesis, cloning, chromosomal 'walking' and the creation of gene fusions. They are also useful alternatives to autonomously-replicating plasmids for transferring point mutations from cloned DNA to the chromosome and vice versa, and they provide a way to map cloned genes without known genetic markers. In addition, integrational vectors afford the possibility of amplifying the copy number of a cloned gene in a controlled fashion, which under some circumstances can yield important information about its regulation.

In developmental studies of bacteria, proteins specific for certain stages of differentiation have often been characterized before their structural genes have been identified, eg protein S from the spore coat of M. xanthus, the small acid-soluble polypeptides (SASP) associated with the DNA in the spore core of B. subtilis and the spore coat polypeptides of B. subtilis (Losick, 1986). Such genes have been cloned in E. coli using strategies based on working backwards from the protein product of the genes to the genes themselves. The techniques used in this 'reverse genetics' include immunodetection of the expression of the cloned genes in E. coli and the use of synthetic oligonucleotides (based on prior knowledge of amino acid sequences of the protein product of the gene) as hybridization probes.

1.2.3 Combining the classical and new genetics

As described above there are a large number of genetic tools of considerable power available for the study of development in microorganisms. There are, however, still some requirements that must be met by the underlying genetics of the organism under study. Minimally, the organism must be amenable to manipulation to the extent that double mutants can easily be constructed: this means that a transposon/generalized transduction system should be available. The genetics must allow the backcrossing of pseudorevertants to wild-type and the recovery of suppressors: this too is easily achieved with transposon/transduction systems since a mutation linked to a transposon can be transferred away from its suppressor by selecting the drug resistance. If the full range of methods is to be used, then a way must be found to return cloned DNA fragments to the organism (by DNA transformation or conjugation at least) and to have them maintained there either extra-chromosomally or through chromosomal integration. To make best use of in vitro mutagenesis methods, a way is needed to replace normal alleles of genes with mutant alleles carried on plasmid or phage.

Genetic analysis of any process cannot proceed until mutants specific to that process are available. Generally these will still have to be isolated in the first instance by direct screening. However, once a few mutants have been obtained, several additional routes, possibly more direct and efficient, become available. Using transposons linked to the genes of interest, one can saturate the gene and surrounding region by localized mutagenesis; without mutagenesis of the entire genome. One can

Organism	<u>Bacillus</u>	<u>Caulobacter</u>	<u>Streptomyces</u>	Myxobacteria
Temperate phage	p11, ϕ 105, seb	/	ϕ C31, TG1	Mx8
Generalized transduction	pBS1 coarse mapping	ϕ Cr30	<u>S. fraediae</u> <u>S. venezulae</u>	P1, Mx4, Mx8, Mx9
Specialized transduction	SPb	-	ϕ c31 modified	-
Lysogenic phage	partial diploids for complementation	/	/	P1/
Transformation:	/	-	/	/
naked linear DNA	/	-		
	fine mapping			
plasmid DNA		-	/PEG	electroporation
Protoplast fusion	/PEG	-	/	-
			lysozyme/PEG	
Indigenous plasmids	/	/	/	Single stranded DNA and a circular phage
		(not useful)		
Transfer of broad host range plasmids	/	/	/	RP4 (integrates)
		<u>IncP</u>		
Chromosome mobilization	-?	/ RP4	/	-
Integrative vectors	/	/	ϕ C31 derivatives	/
Transposon mutagenesis	<u>Tn917</u>	<u>Tn5</u> , <u>Tn7</u>	<u>Tn4560</u> <u>Tn5</u> + <u>Streptomyces</u> promoters	P1::Tn5
Gene (<u>lacZ</u>) fusions	/	/	/ (ϕ C31)	/
Chemical mutagenesis	/	/	/	/
Cloning of developmental genes	/	/	/	/

Organism	<u>Bacillus</u>	<u>Caulobacter</u>	<u>Streptomyces</u>	Myxobacteria
Genes returned to cells	/	/	/	/ merodiploids
Isolation of extragenic suppressors	/	-	-?	-
Gene map	/	/	/	limited restriction map

Table 1.2.3 Availability of genetic techniques for developing prokaryotes

also clone DNA fragments that complement the mutation of interest, and use the clones to make more mutations, including completely defective alleles (eg deletion of nonsense mutations) even when the gene function is an essential one. It is useful, of course, to find cold-sensitive and heat-sensitive alleles of interesting genes in order to carry out reciprocal-shift experiments.

One can proceed to isolate pseudorevertants, either directly in the genome or in cloned DNA fragments. The genes identified as giving rise to suppressors are amenable to further analysis, either because the suppressors have an additional phenotype, or because the suppressors are in the form of recombinant clones.

Finally, if there is the suspicion that a particular protein might be involved in the process of interest, one can use recombinant DNA methods to isolate the structural gene(s) encoding this protein and make mutations therein and, using an allele replacement system, make mutations in the genome whose phenotype can be assessed.

Thus the way in which the recently-developed cloning and gene-manipulation technique have the greatest impact on the study of development in bacteria is by facilitating the application of traditional genetic approaches.

Four of the developing prokaryotes are currently amenable to these genetic analyses. These are Bacillus spp., M. xanthus, C. crescentus and Streptomyces (S. coelicolor and S. lividans). Table 1.2.3 summarizes the systems for genetic analysis of these organisms.

1.3 Genetics of the purple non-sulphur bacteria

(Nomenclature changes: in a rearrangement of the species and genera of the prototrophic non-sulphur bacteria (Imhoff et al. 1984), Rhodopseudomonas capsulata and Rhodopseudomonas sphaeroides became Rhodobacter capsulatus and Rhodobacter sphaeroides respectively. The latter names are used in this work).

1.3.1 Genetic organization of members of the Rhodospirillaceae Genomes

Among the Rhodospirillaceae the DNA base compositions vary between 62 and 72 mole % G+C. The sizes of genomes of purple non-sulphur bacteria are estimated to be 2.9×10^9 D for Rhodobacter capsulatus (Yen et al. 1979); 2.1×10^9 D for Rm. vannielii (Potts et al. 1980) and 1.6×10^9 D for Rhodobacter sphaeroides (Gibson and Neiderman, 1970). These values are close to those of other bacteria.

Extrachromosomal DNA

Indigenous plasmids

Extrachromosomal genetic elements (plasmids) are prevalent amongst bacteria, where they specify a diversity of biological functions (Hardy, 1981). There have been a number of reports describing the presence of plasmid DNA in purple non-sulphur bacteria. Extrachromosomal DNA was first detected in photosynthetic bacteria by Suyama and Gibson (1966). Subsequent findings confirmed and extended these initial observations and to date plasmid DNA has been identified in a number of species including Rb. sphaeroides (Suyama and Gibson, 1966; Gibson and

Neiderman, 1970; Saunders et al. 1976; Pemberton and Tucker, 1977; Tucker and Pemberton, 1978; Fornari et al. 1984; Matsunaga et al. 1986), Rb. capsulatus (Hu and Marrs, 1979), and Rhodospirillum rubrum (Kohl et al., 1983). In the majority of cases, multiple classes of plasmid DNA have been detected. Molecular weights of the plasmids fall within the range from 5×10^6 to 10^8 D.

To date, the identity of genetic determinants carried by plasmids in the purple non-sulphur bacteria remains cryptic. There has been some speculation that plasmid DNA may play a role in specifying the photosynthetic apparatus (Gibson and Neiderman, 1970; Saunders et al. 1976; Kohl et al., 1983, 1984). However plasmids are generally dispensable (Novick, 1969; Novick et al. 1976) which tends to reduce the credibility of this suggestion. Nevertheless, there does seem to be some relationship between plasmid profile and photosynthetic competence in Rb. sphaeroides at least (Saunders et al. 1976; Nano and Kaplan, 1984). Plasmid rearrangement is a general phenomenon which can occur spontaneously in Rb. sphaeroides 2.4.1 and shows a high correlation with a photosynthesis-minus phenotype. In contrast Magnin et al. (1987) and Willison et al. (1987) found that strains of Rb. capsulatus lacking endogenous plasmids were photosynthetically-competent and grew autotrophically, showing that all essential genes for these two processes are located on the chromosome. Two further lines of evidence tend to indicate that the genes for photosynthesis are not normally plasmid-borne in Rb. capsulatus: conjugative studies linking genes for photosynthesis to typical chromosomal markers (Marrs, 1981) and the use of cloned photosynthesis genes to probe for homology amongst restriction endonuclease digests of chromosomal and plasmid DNAs. Nano and Kaplan (1984) suggested that the observed coincidence of plasmid rearrangements

and mutations affecting the photosynthetic apparatus may reflect the presence of an endogenous genetic element in Rb. sphaeroides 2.4.1 that is capable of affecting both plasmid stability and photosynthetic phenotype.

Other possible functions for the plasmid DNA of the purple non-sulphur bacteria include resistance to antibiotics or heavy metals, production of toxins, and conjugation ability. These possibilities are discussed in detail elsewhere (Saunders et al. 1976; Saunders, 1978). There have been several reports of antibiotic-resistance amongst these organisms and the resistance has been attributed to plasmid-borne genes in Rb. capsulatus (Weaver et al. 1975) and to a naturally occurring viral R-plasmid (P ϕ 6P) in Rb. sphaeroides (Pemberton and Tucker, 1977). The molecular sizes of some of the plasmids of the Rhodospirillaceae are consistent with those of conjugative plasmids (generally >20 megadaltons). So far, however, unequivocal genetic exchange mediated by indigenous plasmids has not been demonstrated. If such plasmids are shown to promote their own transfer by conjugation, they may be more effective than 'foreign' plasmids at mobilizing chromosomal genes. Some 'foreign' plasmids are already capable of mediating chromosome transfer in members of the Rhodospirillaceae.

Exogenous plasmids

Because of their broad host-range, multiple antibiotic-resistance, plasmids of the incompatibility (Inc) groups P-1, W, N and X were likely candidates for setting up conjugation systems in the purple non-sulphur bacteria (Saunders, 1978). In 1977, Sistrom reported the transfer of the Inc P-1 plasmid R68.45 into strains of Rb. sphaeroides and

Rhodopseudomonas gelatinosa. In the following five years a variety of naturally occurring Inc P, W and W plasmids were transferred into both Rb. sphaeroides and Rb. capsulatus (Miller and Kaplan, 1978; Saunders, 1978; Pemberton and Bowen, 1981; Pemberton et al., 1983). Such plasmids transfer freely between various strains of Rb. sphaeroides and Rb. capsulatus and from these into a range of other organisms, eg E. coli, Alcaligenes spp and Pseudomonas spp (Pemberton et al., 1983). These plasmids are relatively stable in strains of rhodopseudomonads. Subsequently a number of systems based on those basic broad host-range replicons have been developed for chromosome transfer, cloning, the introduction of transposable genetic elements and the generation of R-prime elements. The use of such vectors as agents of genetic exchange is discussed below. The range of broad host-range plasmids available for most Gram-negative bacteria has been reviewed by Franklin (1985).

Bacteriophages and Bacteriocins

The value of transduction in the provision of fine-structure maps and the construction of mutant strains is indisputable. The quest for corresponding phage-mediated gene transfer systems within the Rhodospirillaceae has promoted studies on the virology of these organisms and this is reviewed below. To date, all attempts to find a high frequency generalized transducing phage for any member of the group have failed. Surveys (Guest, 1974; Wall et al., 1975) revealed that representatives of the Rhodospirillaceae produce bacteriocins. Rb. sphaeroides and Rhodopseudomonas palustris exhibit few interspecies-specific inhibitory interactions. Greatest inhibitory activity, both interspecies and intraspecies, was exhibited by strains of Rb. capsulatus. In addition, purple non-sulphur bacteria produce

antimicrobial substances which are not bacteriocins but are metabolites extractable with organic solvents (Kapsari and Klemme, 1977). Such antibiotic effects produced by Rb. sphaeroides (strain 1e7) were restricted to certain Gram-positive bacteria, eg B. subtilis whereas those produced by Rb. capsulatus (strain FC101) appeared to be non-specific. Bacteriocins are of interest genetically since their production is often determined by transmissible plasmids which could be exploited as vehicles of genetic exchange.

1.3.2 Isolation of mutants

The genetics of photosynthetic bacteria began over thirty years ago with the isolation of mutants of Rb. sphaeroides that had defects in the synthesis of carotenoid pigments and the elucidation of the pathway of carotenoid synthesis (Griffiths and Stanier, 1956). Until 1974, when Marrs discovered the gene transfer agent (GTA) of Rb. capsulatus, the application of genetics to research on the photosynthetic bacteria was limited to this sort of mutant analysis as no method of gene transfer was known. Over the years a wide range of mutants have been isolated in various members of the Rhodospirillaceae and have proved invaluable as research tools for probing the physiological processes of these bacteria. Saunders (1978) surveyed the range of mutants including resistant mutants, auxotrophs, pigment mutants, temperature-sensitive mutants and morphological mutants. These have facilitated studies in electron transfer processes, nitrogen fixation, pigment biosynthesis, membrane development and differentiation. Marrs (1978a) considered the applicability of mutants to the resolution of photosynthetic mechanisms, and the range of mutants affecting photosynthesis was reviewed by Saunders (1982).

1.3.3 Genetic transfer and tools for genetic manipulation

Transduction

The value of phages so far found for Rhodospirillaceae as agents of genetic exchange remains limited at present. Only in a few instances has phage-mediated gene transfer been demonstrated. A temperate phage RS-2, exhibiting low level transducing activity for a variety of genetic markers in Rb. sphaeroides, was isolated by Kaplan and colleagues (Marrs, 1978b). The same group introduced the coliphage P1 into Rb. sphaeroides making P1-promoted gene transfer a possibility (Marrs *et al.* 1977). The potential in developing either of these two phage as tools for genetic transfer has not been realized. Pemberton and Tucker (1977) demonstrated effective transduction of a penicillin-resistance determinant between strains of Rb. sphaeroides mediated by the viral R-plasmid R ϕ 6P. However, attempts to perform generalized transduction with R ϕ 6P failed.

Capsduction

The first genetic transfer mechanism discovered for a phototrophic bacterium was capsduction in Rb. capsulatus (Marrs, 1974). Capsduction resembles transduction in that phage-like particles are the vectors that carry DNA from donor to recipient cells, yet the process is fundamentally different from transduction in that no virus is associated with particle production. The particles that serve as vectors for capsduction are called gene transfer agents (GTA) which resemble small, tailed bacterial viruses, albeit much smaller than any known phage of similar complexity. GTA particles can apparently promote transfer of any

region of the bacterial genome, including plasmid DNA with equal probability, and frequencies of recombination approach 10^{-3} per recipient cell. Biological assays, and physical tests, indicate that there is no viral activity associated with GTA production. GTA preparations are not infectious, since strains of Rb. capsulatus that do not produce GTA naturally do not begin to produce it after GTA exposure. The distribution of GTA amongst isolates of Rb. capsulatus is widespread. In one survey 19 out of 33 wild-type strains tested were capable of GTA production and 25 showed competence as recipients (Wall *et al.* 1975). GTA production is restricted to Rb. capsulatus.

Mapping and strain construction are both possible using capsduction. The DNA fragments introduced by GTA are not capable of autonomous replication, so recombination between the incoming fragment and the resident chromosome must occur for a stable genetic event to occur. The 5kb fragment can introduce about five genes at a time, and from this fragment a piece may be integrated. Since the amount of DNA transferred is small, strains constructed via GTA differ only minimally from the original recipient phenotype. Several genetic transfers can occur simultaneously and independently in one recipient cell if several donor particles are taken up. Capsduction does not result in any detectable immunity to subsequent GTA uptake, and many strains are self-fertile, so strains may be constructed one step at a time by a series of GTA-mediated crosses (Marrs, 1978b).

GTA mapping was initially applied to the photopigment (bacteriochlorophyll and carotenoids) region of the Rb. capsulatus chromosome (Yen and Marrs, 1976). The maps obtained are very fine structure maps, resolving the relative positions of many mutations

within each gene and are also remarkably additive and internally consistent.

Since the GTA particles contain fragments of the donor organism, apparently cut in uniform length and packaged at random, that cannot replicate when deposited in the recipient cytoplasm, the system is useful in interposon mutagenesis. If an interposon can fit within the GTA head and still carry enough flanking DNA to produce regions of homology for recombination, capsduction provides a very simple means for introducing the interposon into the recipient genome. This technique has been successfully applied in the study of genes for photosynthesis and nitrogen fixation in Rb. capsulatus (Scholnik and Haselkorn, 1984; Youvan et al. 1985). Absence of transducing phage and the limited size of DNA fragment transferred by GTA stimulated the search for conjugative transfer mechanisms.

Conjugation

The first report of a conjugation system for a phototrophic bacteria was the transfer of R68.45 (an R-factor variant with enhanced sex-factor activity) from P. aeruginosa to Rb. sphaeroides (Sistrom, 1977). Rb. sphaeroides strains carrying R68.45 served as conjugative donors to other strains of this species. All of the chromosomal markers tested were transferred at frequencies from 10^{-4} to 10^{-7} recombinants per donor cell. The basis for enhanced chromosome mobilization of R68.45 over R68 is a duplication of the 2.1kb 1S21 sequence. A possible explanation for the inability of particular Inc P plasmids to mobilize the chromosome of Rb. sphaeroides efficiently could be the lack of a specific region of genetic homology between the plasmid and the host chromosome. Such

PLASMID(S)	Inc. Gp.	Organism	Transfer (+/-)	Comments	Reference
R1822(RP1, R68, RK2)	P	<u>Rb.sphaeroides</u>	+	unstable	Olsen and Shipley, 1973
	P	<u>Rs.rubrum</u>	+	unstable	
R68.45	P	<u>Rb.sphaeroides</u>	+	transfer on solid medium stable, low level	Sistrom 1977
		<u>Rd.gelatinosa</u>	+	c.m.	
RP4	P	<u>Rb.sphaeroides</u> <u>Rb.capsulatus</u>	+	maximal transfer on solid medium	Miller and Kaplan, 1978
RP1	P	<u>Rb.capsulatus</u> SB1003	+		Jasper <u>et al.</u> 1978
R68.45	P	<u>Rb.capsulatus</u>	+		Marrs <u>et al.</u> 1977
RP4/R68.45		<u>Rb.sphaeroides</u> RS630	+)	low level c.m. Tucker and Pemberton 1979b
R751/R702	P	<u>Rb.sphaeroides</u> RS630	+)	
R388	W	<u>Rb.sphaeroides</u> RS630	+)	
S-a	W	<u>Rb.sphaeroides</u> RS630	+)	
R1/R1-16	FII	<u>Rb.sphaeroides</u> RS630	-		
R40a/R75b	C	<u>Rb.sphaeroides</u> RS630	-		
R64	I	<u>Rb.sphaeroides</u> RS630	-		
RP4::Mu _{gts} 62	P	<u>Rb.sphaeroides</u>	+	Mu phage produced	Tucker and Pemberton 1979 a
RP1/R68.45		<u>Rb.capsulatus</u>	+	low level c.m.	Yu <u>et al.</u> 1981
RP4::Mu _{gts} 61	P	<u>Rb.capsulatus</u>	+	low level c.m.; Mu phage produced	
pLM2	P	<u>Rb.capsulatus</u>	+	c.m. -10^{-7}	Marrs, 1981
pBLM2	P	<u>Rb.capsulatus</u>	+	pBLM2 + chromosomal insert c.m. 6×10^{-4}	
pRPS404	P	<u>Rb.capsulatus</u>	+	46 kb insert from pBLM2	

PLASMID(S)	Inc. Gp.	Organism	Transfer (+/-)	Comments	Reference
RP1	P	<u>Rb.sphaeroides</u>	+	c.m. 10^{-3}	Pemberton & Bowen, 1981
pKT230	Q	<u>Rb.sphaeroides</u>	+	RSF1010 derivatives	Bagdasarian et al. 1981
pKT231	Q	<u>Rb.sphaeroides</u>	+	mobilized by	
pKT247	Q	<u>Sb.sphaeroides</u>	+	conjugative vector	
	Q	<u>Rhodopseudomonad</u>	+		Barth et al. 1981
RH2/R751	P	<u>Rs.rubrum</u>	+		Saunders 1982
pRR292/290	P	<u>Rb.capsulatus</u>	+	cloning of <u>nif</u> genes	Avtges et al. 1983
pRK290	P	<u>Rb.capsulatus</u>	+	gene bank mobilized by pRK2013	Klug & Drews, 1984
pUI108/109	Q	<u>Rb.sphaeroides</u>	+	<u>in vitro</u> β -gal fusions	Nano et al. 1984
pAS-9	P/Col E1	<u>Rb.sphaeroides</u> 2R	+		Zinchenko et al., 1984
RSF1010	Q	<u>Rb.sphaeroides</u> 2R	+		
pBR325/pML 21	Col E1	<u>Rb.sphaeroides</u> 2R	+	co-integration with mobilizing plasmid RecA	
pLAFRI	P	<u>Rb.capsulatus</u>	+	gene bank	Avtges et al. 1985
pTH10(RP4 ^c)	P	<u>Rb.capsulatus</u>	+		Willison et al., 1985
pRK404	P	<u>Rb.capsulatus</u>	+	expression vector using cellulase genes	Johnson et al., 1986

Table 1.3.3a Use of conjugative plasmids in the purple non-sulphur bacteria.

(c.m. = chromosome mobilization)

homology might well be provided through insertion sequences or transposable genetic elements. On this basis the hybrid plasmid RP4::Mu cts was introduced to Rb. sphaeroides (Tucker and Pemberton, 1979a) and Rb. capsulatus (Yu et al. 1981). The Mu genome was expressed in both species but frequencies of transfer of chromosomal genes were low. A similar system is RP1::Tn501 which is an Inc P plasmid with enhanced chromosome-mobilizing ability due to the presence of the mercury-resistance transposon. RP1 produced about 10^{-8} recombinants per donor whereas RP1::Tn501 produced between 10^{-3} and 10^{-7} depending upon the selected marker. This system was used to produce the first chromosomal-scale map for a photosynthetic bacterium - Rb. sphaeroides (Pemberton and Bowen, 1981). Another derivative of RP1 with enhanced chromosome mobilization was pBLM2 (Marrs, 1981). This plasmid contained the same 2.1kb IS21 insertion as R68.45 and promoted the formation of up to 6×10^{-4} recombinants per donor in Rb. capsulatus. R-prime derivatives were found amongst the progeny of pBLM2-mediated crosses at a frequency of about 10^{-6} per donor. This indicated that pBLM2 could integrate into the genome of Rb. capsulatus. R-primes were recognisable in this system because the map of the photopigment region made it possible to construct crosses in which rare progeny could be detected on the basis of colony colour. These R-primes which were transmissible to non-photosynthetic species and back, represented recombinant DNA clones created in vivo. They have been valuable, as a source of the photosynthesis genes and for the construction of merodiploids, making it possible to perform cis-trans complementation tests for defining boundaries of genes, and to define their dominant relationships.

Recently Willison et al (1985) used pTHIO (RP4 derivative which is temperature sensitive for maintenance in E. coli) to mobilize the

Rb. capsulatus chromosome at a rate of 10^4 times greater than when using RP4. Transfer was shown to occur from multiple origins. This R plasmid-mediated conjugation system was a major advancement in the genetics of Rb. capsulatus permitting long-range mapping as an alternative to the fine-structure mapping given by GTA. The use of conjugative plasmids in the genetics of the purple non-sulphur bacteria (Rb. sphaeroides and Rb. capsulatus) is summarized in Table 1.3.3a.

Transformation

Table 1.3.3b summarizes the successful attempts to carry out transformation with Rb. capsulatus and Rb. sphaeroides. It appears that the purple non-sulphur bacteria are generally refractory to transformation. Possible explanations for this have been discussed elsewhere (Saunders, 1978; Saunders, 1982). No useful transformation system has been reported for Rb. capsulatus so other methods are currently used to introduce exogenous DNA into this species. The helper effect of phage R ϕ 9 particles on R ϕ 6P DNA-uptake by Rb. sphaeroides (Tucker and Pemberton, 1980) seems to result from an interaction between the R ϕ 9 virus and the cell surface. This system has potential for transfer of recombinant DNA plasmids into Rb. sphaeroides but it has not been applied towards that end.

Fornari and Kaplan (1982) developed a method (treatment with 500mM Tris followed by 100mM CaCl_2 and 20% PEG600) for introducing plasmid DNA into Rb. sphaeroides. Small non-conjugative plasmids were transferred directly at frequencies as high as 10^{-5} per cell.

Matsunga *et al* (1986) described the recovery of Ap-resistant

Organism	DNA	Methods	Frequency per cell	Reference
<u>Rb. capsulatus</u>	ColEI::Tn5		10^{-9}	Jasper <i>et al.</i> 1978
<u>Rb. sphaeroides</u> RS6143	phage Rφ6P carrying β <u>lac</u> lysogens formed	helper phage mediated Rφ9	2×10^{-5}	Tucker and Pemberton, 1980
<u>Rb. sphaeroides</u>	pU181 (RSF 1010/pBR322 hybrid)	500 mM Tris then 100 mM CaCl ₂ and 20% PEG	10^{-5}	Fornari and Kaplan, 1982
<u>Rb. capsulatus</u>		spheroplasts treated with divalent cations	v. low	Saunders, 1982 (c.f. Marrs pers. comm.)
' <u>Rb. sphaeroides</u> '	pURDA (M13 derivative-hybrid with indigenous plasmid)	?	$2.7 \times 10^3 \mu\text{g DNA}^{-1}$	Matsunga <i>et al.</i> 1986

Table 1.3.3b Transformation of purple non-sulphur bacteria

transformants of a marine photosynthetic bacterium tentatively assigned to the species Rb. sphaeroides. Transformants occurred at 2.7×10^3 colonies per μg of pURDA DNA (pURDA was a hybrid plasmid consisting of the M13-derived plasmid pUC13 and an indigenous plasmid). No details of the transformation techniques used were published.

Transposon mutagenesis

With the construction of transposon delivery vectors for mutagenesis (eg pJB4JI, Beringer et al. 1978; pSUP2021, Simon et al. 1983a,b) it was clear that these systems would be applicable to the genetics of photosynthetic bacteria. Simon et al. (1983a) transferred pSUP2021 (ie pSUP202::Tn5) to Rb. capsulatus and Weaver and Tabita (1983) successfully used pJB4JI to isolate mutants of Rb. sphaeroides. Kaufmann et al. (1984) found that pJB4JI was stably maintained in Rb. capsulatus and therefore not useful for transposon mutagenesis in this species. Nevertheless, they found that pACY184::Tn5 was not maintained but the frequency of transposon-induced mutations was low and pSUP2021 was not maintained in Rb. capsulatus and gave rise to 3×10^{-5} Km-resistant transconjugants per donor. The pSUP2021 plasmid, with the mobilizing E. coli strain S17-1, has been used to isolate a variety of Rb. capsulatus mutant types in recent years (Hudig et al. 1986; Wright et al. 1987; Klipp et al. 1988).

With the isolation of specific genes it became possible to carry out directed transposon mutagenesis. The transposon is inserted into the cloned gene of interest and transferred back to the source organism. The transposon inserts site-specifically into the chromosome by homologous recombination. This technique has been applied to the analysis of the

Organism	Plasmid	Reference
<u>Non-specific insertion</u>		
<u>Rb.capsulatus</u>	ColEI::Tn5	Jasper <i>et al.</i> , 1978
<u>Rb.sphaeroides</u>	pJB4JI	Berlinger <i>et al.</i> , 1978
<u>Rb.sphaeroides</u>	RP1::Tn501	Pemberton & Bowen, 1981
<u>Rb.capsulatus</u>	pSUP202::Tn5	Simon <i>et al.</i> , 1983a
<u>Rb.sphaeroides</u>	pJB4JI/RK2 ^{ts} ::Tn5	Weaver and Tabita, 1983
<u>Rb.capsulatus</u>	pJB4JI (maintained)	Kaufman <i>et al.</i> , 1984
<u>Rb.capsulatus</u>	pACY184::Tn5	
<u>Rb.capsulatus</u>	pSUP202::Tn5	
<u>Rb.capsulatus</u>	pSUP202::Tn5 (strain S17-1)	Hudig <i>et al.</i> , 1986
<u>Rb.capsulatus</u>	pSUP202::Tn5 (strain S17-1)	Wright <i>et al.</i> , 1987
<u>Rb.capsulatus</u>	pSUP2021 (strain S17-1)	Klipp <i>et al.</i> , 1988
<u>Site-directed insertion</u>		
<u>Rb.capsulatus</u>	pVCT1415 ColEI::Tn7	Youvan <i>et al.</i> , 1982
<u>Rb.capsulatus</u>	pRPS404::Tn5.7	Youvan <i>et al.</i> , 1983
<u>Rb.capsulatus</u>	pRPS404::Tn5.7	Zsebo and Hearst, 1984
<u>Rb.capsulatus</u>	pRPS404::Tn5	Biel, 1986
<u>Rb.sphaeroides</u>	pSUP202::rbcL::Tn5 pSUP202::rbcS::Tn5	Falcone <i>et al.</i> , 1988

Table 1.3.3c Transposon mutagenesis of purple non-sulphur bacteria

photosynthetic genes of Rb. capsulatus (Youvan et al. 1982; Zsebo and Hearst, 1984) and the ribulose biphosphate carboxylase/oxygenase genes of Rb. sphaeroides (Falcone et al. 1988). The use of transposon technology in Rb. capsulatus and Rb. sphaeroides is summarized in Table 1.3.3c.

Interposon mutagenesis

A very similar technique to directed transposon mutagenesis is interposon mutagenesis which involves the introduction of marker-bearing insertions into any cloned segment of DNA. The interposon is a stretch of DNA coding for a selectable marker, flanked by useful restriction sites, and ideally containing transcription and translation terminators in each orientation. Youvan et al. (1985) have listed commonly used interposons. Perhaps the most useful interposon is the 2.0kb Ω fragment (Prenkti and Krisch, 1984); which was constructed to have the appropriate terminators in both directions and which codes for spectinomycin and streptomycin resistances. The insertion sites may be points (single restriction site) or deletions (2 or more restriction sites). In Rb. capsulatus the GTA can be used to deliver the DNA back to the chromosome by homologous recombination. In Rb. sphaeroides site-specific insertion mutations have been constructed by introducing the modified gene on an unstable plasmid (eg pSUP202). Such site-specific mutations have been constructed in numerous photosynthetic genes with relatively high frequencies in both Rb. capsulatus Davidson and Daldal, (1987) and Rb. sphaeroides (Davis et al. 1988). Additional applications of interposons are the construction of transcriptional fusions and interposon mapping (Scholnik and Marrs, 1987).

Site directed mutagenesis

This is the study of a particular site in a protein by the introduction via genetic engineering of all possible amino acids, one at a time, at that site. Such studies are currently under way using pigment-binding proteins of the photosynthetic apparatus of both Rb. capsulatus and Rb. sphaeroides (Bylina et al., 1986).

Expression vectors

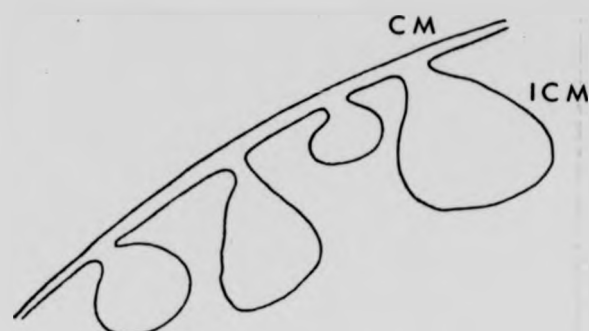
The E. coli lac system was first exploited in the molecular genetic analysis of Rb. sphaeroides by Nano et al. (1985); expression of the lac operon dependent upon transcriptional or translational fusions of Rb. sphaeroides DNA to lacZ provides a powerful genetic tool both to study the expression of specific genes and to select for mutations that affect expression of various gene fusions. A variety of plasmid constructions based on the RSF1010 replicon that facilitate cloning and the construction of translational lacZ fusion have recently been prepared (Kiley and Kaplan, 1988 citing Tai et al. submitted for publication). Similar expression vectors have been constructed for Rb. capsulatus (Johnson et al., 1986). These are based on the broad host-range plasmid pRK404 and use the promoter region of the puf operon from Rb. capsulatus to drive transcription. Either transcriptional or translational fusions can be created, and expression is regulated by oxygen.

1.3.4 Photosynthesis in purple non-sulphur bacteria

Members of the purple photosynthetic bacteria are capable of growth by aerobic and anaerobic respiration, fermentation and anoxygenic

photosynthesis. In general, when growing chemoheterotrophically, these organisms have a typical Gram-negative cell envelope and growth is supported by aerobic respiration. When oxygen is removed from such a culture, a series of events is triggered which results in the differentiation of the cytoplasmic membrane through a process of invagination into specialized domains which comprise the photosynthetic intracytoplasmic membrane system (ICM). The ICM is physically continuous with the cytoplasmic membrane but structurally and functionally distinct in that the ICM specifically contains all the membrane components required for the light reactions of photosynthesis. Within the Rhodospirillaceae a variety of types of arrangement of ICM are known (Kelly and Dow, 1986). Rb. capsulatus and Rb. sphaeroides have a vesicular pattern whilst Rhodopseudomonas viridis and Rm. vannielii have a lamellate arrangement with the ICM lying underneath and parallel to the cytoplasmic membrane (Figure 1.3.4a). These organisms provide an excellent model system with which to study both photosynthesis and membrane development. An advantage of studying membrane biosynthesis in facultative photoheterotrophic bacteria is the ability of these bacteria to synthesize photosynthetic membranes in the absence of light and under conditions in which these membranes are otherwise gratuitous for cell growth. Induction of ICM synthesis under defined physiological conditions allows analysis of ICM formation in both wild-type strains and in photosynthetically-incompetent mutants. Moreover, in addition to regulation of ICM synthesis by oxygen tension, the molecular composition and intracellular amount of the ICM is regulated by light intensity. The amount of ICM per cell and the whole-cell specific bacteriochlorophyll-a (Bchl-a) content increase proportionally as a result of decreasing the light intensity used for growth. Regulation of cultural light intensity is also characterized by the differential synthesis of individual

Vesicular



Lamellate

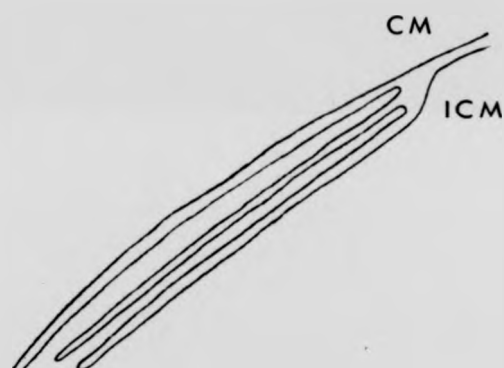


Figure 1.3.4a Vesicular and lamellate patterns of intracytoplasmic membranes of purple non-sulphur bacteria

pigment-protein complexes. Therefore, the inducibility of ICM synthesis by low-oxygen tension has allowed study of the de novo synthesis of a functional biological membrane; by further varying conditions of incident light intensity, questions pertaining to how ICM synthesis and composition are physiologically regulated can also be addressed.

The photosynthetic apparatus of these bacteria consists of four integral membrane pigment-protein complexes plus an ATPase. Three of those complexes are specific to photosynthesis (ie the reaction centre (RC) the two light-harvesting complexes (LHI and LHII)). The remaining one, the ubiquinol:cytochrome c₂ oxidoreductase (bc₁), is common to both respiration and photosynthesis. The most abundant ICM protein complexes are the pigment-protein complexes which contain Bchl-a non-covalently bound to low mol wt (>14kD) proteins. Most species of Rhodospseudomonas and Rhodobacter studied appear to contain the two light-harvesting systems, one with an absorption maximum at 870-890nm (B870 or B890/LHI) and another with two maxima at 800 and 850-860nm (B800-850/LHII). The composition, function and organization of the LH complexes in the photosynthetic membranes of several photosynthetic bacteria have recently been reviewed (Zuber, 1985, 1986). As their name implies, the LH complexes act as antennae to funnel photons to reaction centres (RC), Bchl-protein complexes in which light energy is converted to chemical energy by photo-induced oxidation-reduction reactions. The funneling of photons to the RC occurs by a process of exciton transfer, rather than light energy emission and reabsorption. The arrangement of these Bchl-protein complexes in the ICM phospholipid bilayer must be highly organized to achieve the high efficiency of exciton transfer, since little light is emitted as fluorescence from wild-type strains during photosynthetic growth. In photosynthetic bacteria which contain both LHI

NAME(S)	PROTEIN SUBUNITS	ASSOCIATED PIGMENTS & OTHER GROUPS	FUNCTION(S)	λ (nm)
LHII, Accessory light harvesting complex (B800-850)	α (2mols) β (2mols)	Bchl-a (6mols) Crts (3mols)	variation in light- harvesting ability in response to changing light intensity	B800 /850
LHI, Primary light harvesting complex (B870)	α (1mol) β (1mol)	Bchl-a (2mols) Crts (2mols)	fixed minimum light- harvesting capacity of the photosynthetic unit	B870
RC, Reaction Centre	L (1mol)	'Special pair' Bchl-a (2mols)	Specialized environment in which Bchl can convert energy of	P870
	M (1mol)	'Voyeur' Bchl-a (2mols) Bcpheo (2mols)	excitation into an electron transfer event	P800 760
	H (1mol)		Function unclear; not essential for primary photochemistry	
Cytochrome b/c ₁ complex	cyt c ₁ (1mol) cyt b ₁ (1mol)	2 heme groups and Reiske iron-sulphur protein	involved in cyclic photosynthetic electron flow	
Cytochrome C ₂	cyt c ₂ (1mol)	1 heme group		

Table 1.3.4 Components of pigment-protein complexes of a typical purple
non-sulphur bacterial species

(Molecular ratios cited are for Rb.sphaeroides, Kiley and Kaplan, 1988)

and LHII complexes, the LHI complex appears to be an obligatory intermediate in exciton energy transfer from the LHII complex to the RC complex. Aggregates of LHI complexes surround and possibly interconnect RC complexes within the ICM. These aggregates have been termed the fixed photosynthetic units since the ratio of LHI/RC (15:1) complex is invariant with light intensity. The LHII complexes are peripherally associated around the fixed photosynthetic unit and the amount of LHII complex present varies inversely with incident light intensity. Thus variations in the amount of LHII are responsible for variation in the size of the total photosynthetic units and thus light-harvesting capacity under changing light conditions. The components of the pigment-protein complexes, their absorption maxima and function are summarized in Table 1.3.4.

The absorption of light energy by the RC ultimately results in the photooxidation of a 'special pair' of Bchl molecules (P-870, Table 1.3.4). Thus the RC acts as an energy sink with Bchl in a specialized environment within which it can convert the energy of excitation received from the antennae into an electron transfer event. After exciton absorption the P-870 Bchl enters the singlet excited state and rapidly transfers an electron, probably via one of the 800nm absorbing 'voyeur' Bchls to a single bacteriopheophytin molecule. The initial charge separation is stabilized by electron transfer to the primary quinone acceptor molecule, Q₁.

While the electron from the Bchl dimer is being led to the cytoplasmic side of the membrane, an electron from cytochrome C₂ (cyt C₂) reduces the Bchl₂ 'hole' leaving an oxidized cyt C₂ on the periplasmic side of the membrane. Electrons from Q₁ then cross the membrane to reduce the

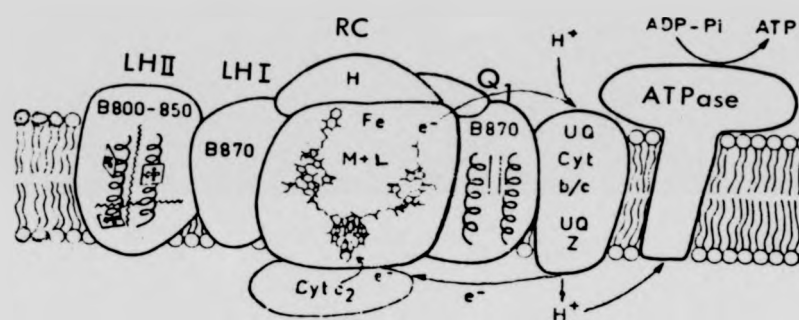


Figure 1.3.4b The relative topology and distribution of ICM components and the kinetic parameters of the cycle photosynthetic electron flow

oxidized cyt C_2 via another membrane-bound complex the bc_1 -oxidoreductase.

Concomitant with this crossing, protons are transported from the cytoplasmic side to the periplasmic side of the membrane, and this proton gradient can then be used to drive cellular ATP synthesis or other energy-requiring processes. The kinetic parameters of this cyclic photosynthetic electron flow and the relative topology and distribution of individual redox centres within the complexes in the ICM bilayer are shown in Fig 1.3.4b.

1.3.5 The genes for photosynthesis

The application of the genetic tools described earlier in conjunction with biophysical techniques has led to considerable advances in the understanding of photosynthesis in the purple non-sulphur bacteria. From genetic studies, it has been shown that the genes encoding the Bchl and carotenoid biosynthetic enzymes and the subunits of the LHI and RC pigment-protein complexes, are clustered in both Rb. sphaeroides and Rb. capsulatus (Kiley and Kaplan, 1988). This was first demonstrated by the isolation of an R-prime from Rb. capsulatus carrying approximately 50kb of Rb. capsulatus DNA (pRPS404) which could complement most mutants deficient in various aspects of photosynthetic function (Marrs, 1981). This plasmid contained the genes for the synthesis of coloured carotenoids from phytoene, Bchl biosynthesis and the structural genes for the RC and LHI polypeptides (Scolnik et al., 1980; Taylor et al., 1983; Youvan et al., 1984a). An analogous R-prime to the Rb. capsulatus pRPS404 has been isolated from Rb. sphaeroides (Sistrom et al., 1984). The isolation of these clusters of photosynthetic genes has permitted

mapping and cloning of most of the structural genes for previously described ICM components (Williams et al., 1983; Youvan et al., 1984a,b; Youvan et al., 1985; Donohue et al., 1986a,b). In the bacteria that have been examined, the structural genes for two of the RC polypeptides, RC-L and RC-M, designated pufL and pufM, respectively, are transcriptionally linked to the two structural genes for the α and β subunits of the LHI complex, pufA and pufB respectively (Belasco et al., 1985; Zhu et al., 1985; Zhu et al., 1986). The structural gene for the third polypeptide in the RC, RC-H (designated puhA) is not linked to the puf operon and maps 50kb away in Rb. capsulatus (Taylor et al., 1983) and Rb. sphaeroides (Kiley and Kaplan, 1988). The intervening region of DNA carries the pigment biosynthesis genes (bch and crt) and these have been mapped and cloned in Rb. capsulatus (Taylor et al., 1983; Zsebo and Hearst, 1984). The structural genes for the LHII subunits α and β are also linked to one another and comprise the puv operon. The genes encoding the subunits of the cyt bc_1 complex have been cloned from both Rb. capsulatus (Youvan and Ismail, 1985) and Rb. sphaeroides (Ashby et al., 1987; Kiley and Kaplan, 1987) and comprise an operon. The gene order is Reiske iron-sulphur protein gene, cyt B and cyt C_1 (the fbp or pet operon). The gene encoding the soluble cyt C_2 (cyxA) has also been cloned and mapped in Rb. sphaeroides (Gebellini et al., 1985) and Rb. capsulatus (Davidson and Daldal, 1987a,b).

Most of the above mentioned genes cloned from Rb. capsulatus and Rb. sphaeroides have also been sequenced, making it possible to make predictions about secondary structure, membrane orientation and roles in binding carotenoid, Bchl and quinone cofactors. This has coincided with the resolution of X-ray crystal structures for the RC of R. viridis (Michel, 1982) and Rb. sphaeroides (Allen and Feher, 1984). Detailed

spectroscopic analysis of the Bchl and quinone intermediates formed during the photochemical reactions is at an advanced stage in Rb. sphaeroides. It is now feasible, using a combination of biophysics and molecular genetics, to study the effects of single amino acid replacements in the structural polypeptides and to correlate these mutations with the physical steps involved in light absorption, charge separation and proton pumping. In addition to the structure-function and bioenergetic aspects of photosynthesis, understanding of the regulation of ICM synthesis (in response to oxygen and light) and the control of photosynthetic gene expression is advancing rapidly in these organisms.

1.3.6 Summary

A variety of genetic techniques have been developed for members of the Rhodospirillaceae (notably Rb. capsulatus and Rb. sphaeroides) primarily for the study of photosynthesis. The most fruitful approach has been the exploitation of exogenous conjugative plasmid technology for the isolation of photosynthetic genes, complementation of mutants and the application of transposon and interposon techniques. In contrast, attempts to develop techniques based upon indigenous genetic entities have been generally unsuccessful. The notable exception is the GTA which is specific for Rb. capsulatus. In addition, purple non-sulphur bacteria are refractory to transformation methods developed in other Gram-negative bacteria. This suggests that it would be most fruitful to try and develop a system of genetic analysis for Rm. vannielii based upon the transfer of exogenous plasmids (broad host-range vectors and transposon delivery vectors).

1.4 Why study *Rm. vannielii*?

1.4.1 The life cycle of *Rm. vannielii*

Rm. vannielii is a phototrophic Gram-negative purple bacterium characterized by the production of ovoid cells linked by integral cellular filaments or prosthecae. It was placed in the order Rhodospirillaceae or the purple non-sulphur bacteria on the basis of its phototrophic style of life and its metabolic capabilities (Pfennig and Truper, 1974). Members of the genus *Rhodomicrobium* have peritrichously-flagellated, stalk-forming cells which multiply by budding and have ICMs parallel to the cytoplasmic membrane. This genus was retained in a recent rearrangement of the taxonomy of the purple non-sulphur bacteria because of its characteristic morphological properties (Imhoff *et al.*, 1984). The life cycle of *Rhodomicrobium* is in contrast to that of other developing prokaryotes in that it can both develop a resting stage (unique angular exospores) as an alternative to vegetative growth and also exhibits events of developmental interest in two different vegetative growth cycles (Figure 1.4.1). In both vegetative cycles single growth-precursors or swarmer cells undergo an obligate sequence of morphogenesis and differentiation (maturation) which results in the swarmer cell becoming a non-motile reproductive cell. The latter synthesizes a prosthecae at one pole and a bud (daughter cell) is synthesized at the distal end of the filament. Before the daughter cell attains maturity it becomes committed to one of three distinct fates, depending upon environmental cues. (1) In the complex cycle it is physiologically separated from the mother cell by the formation of a plug within the filament. This is the first step in chain formation or branched chain formation leading to multicellular arrays. Subsequent sibling production follows a defined set of rules (Whittenbury and Dow,

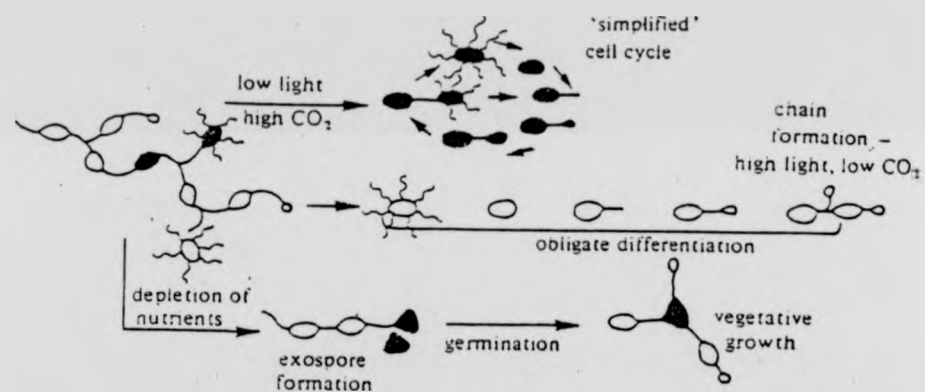


Figure 1.4.1 The life cycle of *Rm. vannielii* exhibits two vegetative growth cycles and an exospore cycle
(from Whittenbury and Dow, 1977)

1977). This cycle is stimulated by conditions of high light and low CO₂ concentration and swarmer cells are produced when the number of cells in a culture or microcolony is enough to cause low light conditions. (2) In the simple cell cycle a motile daughter cell is separated from the mother cell by binary fission, leaving the pole of the prosthecum available for a second round of reproduction. The daughter cell matures and restarts the cycle. No branching occurs and only one prosthecum is synthesized. This cycle is stimulated by conditions of low light and high CO₂ concentration. (3) In the exospore cycle, which appears to be a modification of the complex cycle, up to four exospores are formed and released sequentially from one prosthecum. Sibling production follows the same set of rules as multicellular array formation. Mother cells either produce vegetative cells or exospores but not both. Exospore formation is apparently induced by nutrient depletion.

Large numbers of swarmer cells are produced in mid/late exponential phase of batch cultures expressing the complex cycle due to the decreasing light intensity within the culture. These are easily separated from the multicellular arrays and chains of cells by selective filtration and can subsequently be grown as a synchronous culture. Synchronized swarmer cells incubated phototrophically develop through the obligate sequence that is common to both simple and complex cycles. This sequence of events, known as the 'swarmer cell cycle' has been the focus of developmental studies in Rm. vannielii. The cells pass through a series of well defined morphological stages which can be correlated with key 'landmark' biochemical and physiological events.

1.4.2 Rhodomicrobium as a system for developmental studies

The criteria that make the use of bacteria reasonable as model systems for cell development include: (i) a simple and well defined differentiation pattern which can be studied in synchronized cell populations; (ii) the ability to grow the cells on a defined medium, permitting the correlation of biochemical events with morphological development and (iii) the availability of mutants and a system for the exchange of genetic information (Shapiro et al 1971). Two further desirable characteristics are (iv) the organism should be easy to work with metabolically without major problems in making cell-free extracts or isolating enzymes and (v) the morphological cycle should be subject to nutritional control and it should be possible to grow the organism through a cycle with no morphological changes occurring so that changes associated with differentiation can be separated from those due to cell ageing.

Rhodomicrobium fulfills all of these criteria except for the availability of mutants and a system for the exchange of genetic information. The fulfilment of this third criterion was the aim of the work described here. The other criteria are fulfilled as follows: (1) batch cultures of Rm. vannieli can be easily and selectively synchronized by filtration (Whittenbury and Dow, 1977). Using cultures of up to 20L, filtrates containing about 2×10^7 viable cells ml^{-1} can be prepared within 10-15 min of harvesting. Routinely more than 97% of these are swarmer cells which have experienced negligible physiological stress. Such populations, if incubated anaerobically in the light develop through the obligate series of morphological stages described above; (2) Rm. vannieli grows in a simple pyruvate, malate and mineral salts

medium; (3) work on Rm. vanniellii has proceeded in this department for over a decade and there is much accumulated physiological and biochemical knowledge of this organism; (4) the 'swarmer cell cycle' is clearly under environmental control. It is possible to hold swarmer cell populations in a non-differentiating state by maintaining them in anaerobic/dark conditions. No loss of viability is observed over periods up to 16h and differentiation does not occur. Under exposure to light the population proceeds to differentiate synchronously. This gives the experiments a very powerful control over the initiation of differentiation and provides a population of non-differentiating cells with which the morphology and biochemistry of the differentiating cells can be compared.

A number of other systems fulfil all of these criteria including the availability of mutants and a system of genetic transfer, ie Bacillus, Streptomyces, Myxococcus and Caulobacter. The latter is a 'budding-bacterium' like Rm. vanniellii and they share many common properties. However, Caulobacter has an advanced system of genetic analysis. Rhodomicrobium has been promoted as a model system for the study of differentiation and its photosynthetic membrane system because of several unique characteristics. In Rhodomicrobium, like Caulobacter, differentiation and morphogenesis is an obligate part of the vegetative cell cycle. The swarmer cell cycle offers similar opportunities to study the temporal and spatial control of gene expression as Caulobacter. However the degree to which the initiation of differentiation can be controlled is much greater in Rhodomicrobium and it is much easier to obtain large numbers of non-stressed cells for synchrony. Both Caulobacter and Rhodomicrobium exhibit polar asymmetric growth. In Rhodomicrobium the daughter cell is produced at the same pole as the

prosthecum and actually buds on the end of the prosthecum. A consequence of this is that cell components for the daughter cell must be synthesized de novo. Thus the spatial separation of mother and daughter cell is much more pronounced in Rhodomicrobium than in Caulobacter. In addition Rhodomicrobium exhibits other morphological changes in the formation of chain cells and exospores. Thus it provides opportunity for the study of population heterogeneity, multicellularity and both cell cycle-dependent and independent differentiation.

1.4.3 Rhodomicrobium as a system for studying photosynthesis

The study of photosynthesis and photosynthetic membrane development is well advanced in Rb. capsulatus and Rb. sphaeroides. Rm. vannielii appears to have similar photosynthetic units (LHII and LHI-RC complexes) and the regulation of their synthesis in response to environmental stimuli (light/oxygen) is similar to that in Rb. capsulatus and Rb. sphaeroides (Kelly, 1985). Nevertheless, Rhodomicrobium has some special characteristics which make the study of its photosynthetic system worthwhile. The most fundamental difference is that the ICMs of Rhodomicrobium are lamellate whereas those in the aforementioned organisms are vesicular. In Rm. vannielii, synthesis of the ICM system is integral to the developmental cycle and is one of the differentiation events that has been studied (Kelly, 1985; Kelly and Dow, 1986). One consequence of polar growth is that the ICM complexes must be formed de novo in the daughter cell during differentiation. Thus their synthesis involves temporal and spatial control of gene expression. Thus development of the photosynthetic apparatus can be studied in Rhodomicrobium with the same experimental advantages that were described for other differentiation events above.

1.4.4 Summary of events occurring during differentiation in *Rm. vannielii*

This is a brief summary of what is known about the molecular aspects of differentiation in *Rm. vannielii*. For details of how the information was obtained experimentally the reader is directed to the references given.

Synthesis of proteins in synchronized populations

Gross protein synthesis changes during the swarmer cell cycle with a marked decrease during the period of prosthecum formation (Whittenbury and Dow, 1977). Sequential protein synthesis occurs during synchronous growth. There are quantitative and qualitative changes in protein synthesis during differentiation, compared with protein synthesis in non-differentiating swarmer cells (Porter, 1984; Porter and Dow, 1987). Comparison of the protein profiles from swarmer cells subjected to light and dark regimes revealed differential protein synthesis. Most proteins are specific to the differentiating cell but a few are specific to the inhibited swarmer. In particular one 11.5kD peptide was correlated with the inhibited state (ie dark anaerobic conditions). This peptide was present at low concentration, had a high turnover rate and was actively degraded when the swarmer cells were exposed to light. This protein could play a role in the regulation of initiation of swarmer cell differentiation, ie inhibit differentiation under conditions of light limitation (Porter and Dow, 1987). Kelly and Dow (1986) examined temporal changes in ICM proteins during the swarmer cell cycle, showing that these exhibited a temporal program of synthesis. There was a clear difference between ICM protein synthesis in swarmer cells and chain

cells. These proteins included a 34kD protein, thought to be flagellin, which was only synthesized in swarmer cells and the LHI protein- α (14kD) (Kelly and Dow, 1985a). The latter was not synthesized in swarmer cells and was synthesized during daughter cell formation.

Protein phosphorylation during the swarmer cell cycle has been investigated (Turner and Mann, 1986) and at least 25 proteins are phosphorylated. Phosphorylated proteins were not detected in the swarmer cell prior to differentiation, but as differentiation proceeds a 55kD and a 80kD phosphoprotein became increasingly abundant and several minor phosphorylated species appear. At least three proteins including the 55kD protein showed growth stage-specific patterns of phosphorylation. It remains to be proven that any of these proteins play a regulatory role in differentiation.

RNA synthesis

There is little RNA synthesis in the swarmer cell. Synthesis begins at the initiation of differentiation and there is a large increase in RNA synthesis at 3hr into differentiation (ie onset of daughter cell formation) (Potts and Dow, 1979; Dow *et al.*, 1985). Protein synthesis in swarmer cells, incubated anaerobically in the dark, is dependent upon a rifampicin-sensitive step indicating a dependence on de novo RNA synthesis (Scott and Dow, 1986a). Reproductive cells are sensitive to lower concentrations of rifampicin than swarmer cells (Whittenbury and Dow, 1977). This suggests that RNA-polymerase modification may occur during differentiation. Alternatively it may reflect permeability differences between the two cell types. RNA-polymerase from swarmer cells and differentiating cells were compared (Scott and Dow, 1986b;

Scott et al., 1987) and no differences were observed in the β , β' , α and σ subunits. Amounts of a minor protein associated with RNA polymerase did change during differentiation but it is not known if that protein plays any regulatory function (Scott et al., 1987).

The cell envelope

Scott et al. (1987) proposed that there may be a change in cell envelope permeability properties during differentiation of Rm. vannielii swarmer cells. This proposal was based upon the observations that: (i) the swarmer cell is more resistant to rifampicin than the reproductive cell (Whittenbury and Dow, 1972); Scott et al., 1987); (ii) the swarmer cell is less permeable to nucleotides (Scott and Dow, 1986b) and (iii) the swarmer cell is more resistant to detergent-lysis than the reproductive cell (Scott et al., 1987). These permeability differences cannot be explained by differences in peptidoglycan or LPS structure as no differences were detected between the two cell types (Holst et al., 1986). The reasons for the permeability differences between swarmer cells and reproductive cells are as yet unknown.

Chromosome and DNA synthesis

The genome of Rm. vannielii was characterized by Potts et al. (1980). The base composition was found to be 62% GC and the size of the genome was found to be 2.1×10^9 daltons. This is similar to the size of the E. coli genome. No plasmid DNA was detected. No differences were detected in DNAs isolated from different cell types. However about 5% of the Rm. vannielii genome was found to exhibit rapid renaturation and em. studies showed that this was due to short inverted-repeats of approximately 400

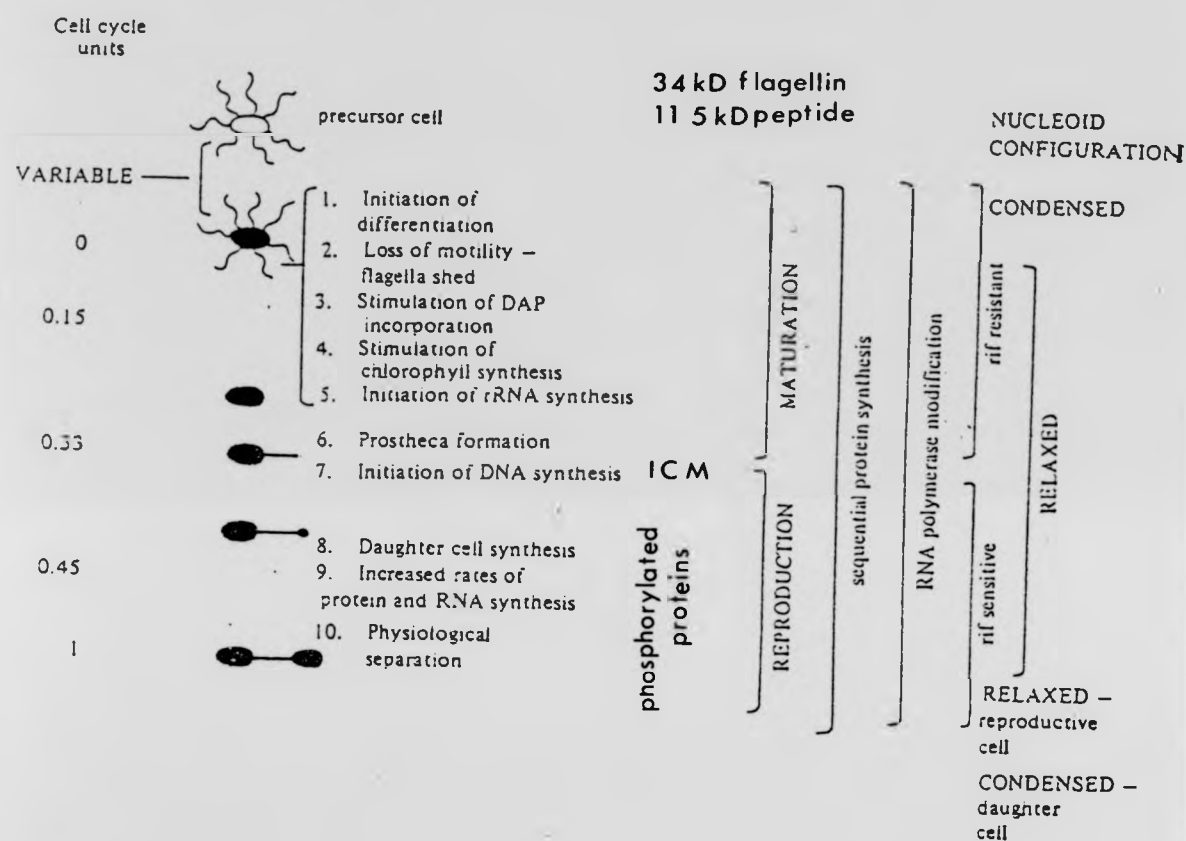


Figure 1.4.4 Summary of the molecular biology of *Rm. vannielii*

base pairs in length (Potts et al., 1980). Although their function is unknown, several suggestions have been made (Potts et al., 1980; Russell and Mann, 1986). The inverted repeat DNA was further characterized by Russell and Mann (1986). Such sequences comprised 7% of the genome and were dispersed throughout the chromosome. They were present in two size-classes (100-700 base pairs and 17-27 base pairs). Southern blotting using the two size classes as probes detected no major differences between restricted DNAs from swarmer and reproductive cells (Russell and Mann, 1986). The conformation of the Rm. vanniellii nucleoid varies during the cell cycle, being highly condensed in the swarmer cell and relaxed in the reproductive cell (Dow et al., 1985). Obvious roles for such changes in differential gene expression have been suggested but remain to be substantiated experimentally. DNA synthesis is not detectable in swarmer cells but is initiated immediately after prosthecum formation (Potts and Dow, 1979). The molecular biology of Rm. vanniellii is summarized in Figure 1.4.4

1.5 Aims of the project

The aims of the work described here were as follows:

- 1) to find methods of generating mutants using classical techniques e.g. chemical mutagenesis or ultraviolet light mutagenesis. This would make if possible to isolate developmental mutants which are essential for the continued study of development/differentiation in Rm.vannielii:
- 2) to find a method of generating mutants using the current transposon delivery systems i.e. Tn₅-insertion mutants; and to demonstrate that such mutant were due to single insertion events;
- 3) to search for indigenous genetic entities (e.g. phage, plasmids), in Rm. vannielii and other available strains of Rhodospirillaceae, which could possibly be developed as vectors for Rm.vannielii:
- 4) in the absence of an indigenous vector, to develop a system of gene transfer based on or using current broad-host-range plasmid technology.

CHAPTER 2

MATERIALS AND METHODS

2.1 Organisms and Media

2.1.1 Rhodomicrobium vannielii and other members of the Rhodospirillaceae

This study focussed upon Rhodomicrobium vannielii Rm5 and derivative strains and these are listed in Table 2.1.1a. These and other members of the Rhodospirillaceae which are detailed in Table 2.1.1b were routinely grown in pyruvate-malate mineral salts (PM) medium (Whittenbury and Dow, 1977) which contained:-

g l⁻¹ distilled water

NH ₄ Cl	0.5
MgSO ₄ · 7H ₂ O	0.4
NaCl	0.4
CaCl ₂ · 2H ₂ O	0.05
Sodium hydrogen malate	1.5
Sodium pyruvate	1.5

The medium was supplemented with 0.1% (w/v) yeast extract for routine growth of all species, including Rm. vannielii although it has no growth factor requirements. For some purposes Rm. vannielii was grown in medium supplemented with yeast extract at concentrations of up to 0.6% (w/v). The yeast extract was added after autoclaving from a filter sterilized stock solution. The pH of the medium was adjusted to 6.8-6.9 with potassium hydroxide (KOH) pellets prior to autoclaving at 121°C for 15 min. After cooling, sterile phosphate buffer was added aseptically to a final concentration of 5mM. For solid media, Difco 'Bacto-agar' was

Table 2.1.1a Strains derived from Rm.vannielii Rm5 and used in this study

Strain	Phenotype description	Reference(s)
Rm5	original isolate	Dow (1974), Whittenbury and Dow (1977)
RB1	spontaneous streptomycin-resistant mutant of Rm5	this study
RB2	spontaneous nalidixic acid-resistant mutant of Rm5	this study
P + S	derivative of Rm5 expressing simplified cell cycle only ('pairs and swarmers')	France (1978)
RB3	spontaneous streptomycin-resistant mutant of P + S	this study
RB4	spontaneous nalidixic acid-resistant mutant of P + S	this study

Table 2.1.1b Other Rm.vannielii strains and other members of the
Rhodospirillaceae used in this study

SPECIES	STRAIN	REFERENCE
<u>Rhodomicrobium vannielii</u>	RV2	
<u>Rhodomicrobium vannielii</u>	RVC3	
<u>Rhodomicrobium vannielii</u>	WRV2	Warwick isolates
<u>Rhodomicrobium vannielii</u>	RV21A	expressing complex
<u>Rhodomicrobium vannielii</u>	RV3/ATCC17100	cell cycle
<u>Rhodomicrobium vannielii</u>	WRV1	
<u>Rhodomicrobium vannielii</u>	RV1	
<u>Rhodopseudomonas acidophila</u>	ATCC 25092	
<u>Rhodospirillum rubrum</u>	NCIB 8255	
<u>Rhodospirillum rubrum</u>	WRR	Warwick
<u>Rhodospirillum rubrum</u>	WRP	isolates
<u>Rhodopseudomonas viridis</u>	ATCC 19567	
<u>Rhodopseudomonas blastica</u>	NCIB 11567	Eckersley and Dow (1980)
<u>Rhodopseudomonas palustris</u>	NCIB 8288	
<u>Rhodobacter sphaeroides</u> "cordata"/81-1	ATCC 33575	Gest <u>et al.</u> (1983)
<u>Rhodobacter sphaeroides</u>	NCIB 8253	
<u>Rhodobacter sphaeroides</u>	green mutant 1719	
<u>Rhodobacter capsulatus</u>	NCIB 8254	
<u>Rhodocyclus gelatinosus</u>	NCIB 8290	
<u>Rhodocyclus tenuis</u>	ATCC 25093	

added (1.5% w/v) before autoclaving and allowed to cool to 60°C before pouring.

2.1.2 Escherichia coli strains

E. coli strains used in this study are detailed in Table 2.1.2 and were routinely grown on Nutrient Broth (NB Oxoid) which was prepared according to the manufacturer's instructions. Nutrient agar NA was NB solidified with (1.5% w/v) Difco 'Bacto-agar'. SOB medium used for efficient transformation of E. coli cells was:

	Per litre distilled water
Bacto Tryptone	20g
Yeast Extract	5g
NaCl	0.5844g
KCl	0.186g

This was autoclaved and then $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and MgCl_2 were added to a final concentration of 10mM from a stock solution of 2M Mg^{2+} ($1\text{M MgCl}_2 \cdot 6\text{H}_2\text{O}$, $1\text{M MgSO}_4 \cdot 7\text{H}_2\text{O}$) which had been filtered.

The minimal medium used for growth of E. coli strains was M9 medium which was prepared as described by Maniatis (1982):

		Per litre distilled water
M9 Salts (10x)	Na_2KPO_4 anhydrous	60g
	KH_2PO_4 anhydrous	30g
	Na_4Cl	10g
	NaCl	5g

Table 2.1.2 *Escherichia coli* strains in this study

Strain	Characteristic	Source	Reference
HB101	F ⁻ , <u>hsdS20</u> (r _B ⁻ , m _B ⁻), <u>rec A13</u> , <u>ara-14</u> , <u>proA2</u> , <u>lacY1</u> , <u>galK2</u> , <u>rpsL20</u> , <u>xyl-5</u> , <u>mtl-1</u> , <u>supE44</u> , λ ⁻	J Hinton (this lab)	Boyer <u>et al.</u> , 1968
HE123	(QD <u>supF</u> , Sm ^r , Nal ^r) F ⁻ , <u>pro</u> , <u>supF</u> , λ ⁻ <u>str</u> , <u>nal</u>	J Hinton (this lab)	Wilson <u>et al.</u> , 1979
S17-1 (pSUP2021)	F ⁻ , <u>thi</u> , <u>pro</u> , (r _k ⁻ m _k ⁺) :: RP4-2 (Tc::Mu) (Km::TnZ)	J Hinton (this lab)	Simon <u>et al.</u> , 1983a
J53 <u>nal</u> (pJB4JI)	F ⁻ , <u>pro</u> , <u>met</u> , <u>nal</u>	J Hinton (this lab)	Beringer <u>et al.</u> , 1978

pH adjusted to 7.4 before autoclaving

M9 minimal medium	10 x M9 salts	100ml
	1M CaCl_2 , 0.1M MgSO_4	10ml) sterilized
	20% (w/v) glucose	10ml) by filtration

2 x Freezing medium was used to store E. coli strains and mating mixtures. This contained:

	Per litre distilled water
K_2HPO_4 anhydrous	126g
Sodium Citrate	0.9g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.18g
$(\text{NH}_4)_2\text{SO}_4$	1.8g
KH_2PO_4 anhydrous	3.6g
glycerol	88g

2.2 Plasmids

The plasmids used in this study are listed in Table 2.2.

2.3 Chemicals

All reagents were of 'Analar' grade or the highest purity available. Media chemicals were generally obtained from Fisons or BDH chemicals. All other chemicals with the exception of those listed below were obtained from the Sigma Chemical Company (London).

Table 2.2 Plasmids used in this study

Plasmid	Inc Gp. Basic Repli -con	Characteristics	Markers for Selection	Source	Reference
pJB4JI	P1	pPHIJI::Mu::Tn ₅	Gm, Sm, Sp, Km	J Hinton	Beringer <i>et al.</i> , 1978
pSUP2021	ColE1	pBR325::mob ⁺ , ::Tn ₅	Ap, Cm, Km	J Hinton	Simon <i>et al.</i> , 1983a
pLG221	I α	ColI-b <i>drd</i> -1, ::Tn ₅	Km	G Boulnois	Boulnois <i>et al.</i> , 1985
pTROY413	P1	pTROY9::lacZ	Tc	J Hinton	Ludwig, 1987
RP4/RK2	P1		Ap, Tc, Km	J Hinton	Thomas, 1981
pRK2501	P1/RK2	Low copy no. mob ⁺	Tc, Km	D Haas	Haas, 1983
pLAFRI	RK2/P1 (pRK290)	Cosmid, mob ⁺	Tc	J Hinton	Friedman <i>et al.</i> , 1982
pGS72	P	Cosmid, mob ⁺	Km, Tc	S Harayama	Selveraj & Iyer, 1985
pKT231	Q	mob ⁺	Sm, Km	C Franklin	Bagdasarian <i>et al.</i> , 1981
pKT240	RSF1010	Promoter probe, mob ⁺	Km	C Franklin	Bagdasarian <i>et al.</i> , 1984
pMMB33/34	RSF1010 /Q	Cosmid, mob ⁺	Km	C Franklin	Frey <i>et al.</i> , 1983
pSF6	W	Cosmid, mob ⁺	Sm, Sp	S Harayama	Selveraj <i>et al.</i> , 1984
pSa151	pSa/W		Sp, Sm, Km, Gm	C Kado	Tait <i>et al.</i> , 1983
pRK2013	ColE1	RK2 <i>trf</i>	Km	J Hinton	Figurski & Helinski, 1979
pBR322	ColE1	multicopy cloning vector	Ap, Tc	Amersham	Bolivar <i>et al.</i> , 1977

Ethidium bromide and phenol came from BDH; propan-2-ol, butan-1-ol, chloroform, iso-amylalcohol and acetic acid were supplied by May and Baker.

Acrylamide, developer D19 and Unifix fixer were obtained from Eastman Kodak. N: N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylene-ethylenediamine (TEMED), ammonium persulphate, sodium dodecylsulphate (SDS), glycine and mercaptoethanol were purchased from Bio-Rad. Unstained molecular weight protein markers for denatured polyacrylamide gel-electrophoresis were obtained from Pharmacia. Radiochemicals, restriction enzymes and pBR322 DNA came from Amersham International-plc. Caesium chloride and glycerol came from Fisons. Gases, Oxygen and Nitrogen were obtained from the British Oxygen Company Ltd (London).

2.4 Buffers

The following buffers were used routinely:

TE pH 8.0 10mM Tris-Cl (pH 8.0)
 1mM EDTA (pH 8.0)

TES/STE 10mM Tris-Cl (pH 8.0)
 100mM NaCl
 1mM EDTA (pH 8.0)

TAE (Tris-Acetate Electrophoresis buffer)

Working solution 0.04 M Tris-acetate
 0.001M EDTA

Concentrated stock (50 x) Per litre

Tris base	108g
85% phosphoric acid	
(1.679 mg/ml)	15.1ml
0.5M EDTA (pH 8.0)	40 ml

TBE (Tris borate Electrophoresis buffer)

Working solution 0.089M Tris-borate
 0.089M boric acid
 0.002M EDTA

Concentrated stock solution (10 x)

Per litre

Tris base	108g
boric acid	55g
0.5M EDTA (pH 8.0)	40ml

Gel loading buffer (x 10)

0.25% bromophenol blue
0.25% xylene cyanol
25% Ficoll (type 400) in H₂O

20 x SSC

Per litre

NaCl	175.3g
Sodium Citrate	88.2g

pH adjusted to 7.0 with NaOH

OLB buffer - this was made up as instructed by Pharmacia

Solution 0	1.25M Tris-HCl
	0.125M $MgCl_2$ pH 8.0

dNTPs	dATP)
	dTTP) 0.1M in TE
	dGTP)

Solution A	1000 μ l solution 0
	18 μ l 2-mercaptoethanol
	5 μ l each of dATP, dTTP, dGTP

Solution B	2M HEPES (titrated to pH 6.0 with 4M NaOH)
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Solution C	Hexadeoxynucleotides (PL # 22-2166-01 Pharmacia)
	50 OD units were suspended in 550 μ l TE to
	give a concentration of 90 OD/ml

OLB Buffer	A:B:C mixed in ratio 10:25:15 (50 μ l)
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2.5 Antibiotics

Antibiotics were prepared as 100 x final concentration stocks and used for E. coli at the concentration shown in Table 2.5. For growth of Rm. vannieli different concentrations of antibiotic had to be used and these will be detailed where relevant.

Tetracycline and Chloramphenol were dissolved in 50% (v/v) ethanol and stored at -20°C. Nalidixic acid was dissolved in 30mM NaOH, filter sterilized and stored at 4°C. All other antibiotics were dissolved in double distilled water, filter sterilized and stored at 4°C.

2.6 Maintenance of cultures

Bacteria of the Rhodospirillaceae were generally maintained as stab cultures in PM agar deeps in 25ml universals. After inoculation from exponential phase liquid culture, they were incubated at 30°C for 3 days under tungsten bulbs and then kept at room temperature. Culture purity was checked by phase contrast microscopy, streaking to obtain isolated colonies on PM agar plates, which were incubated under anaerobic conditions in the light (as described in Section 1.7.1.2) and by spreading 0.2ml of the culture on NA plate, drying and incubating overnight at 30°C.

Alternatively, mutants of Rm. vannieli which were isolated during this study and had to be grown microaerophilically in the dark (Section 2.7.2.1) were stored frozen (to minimize selection of revertants) by (a) adding 0.85ml of an exponential phase culture to a sterile eppendorf tube containing 0.15ml of sterile glycerol and mixing thoroughly by

Table 2.5 Antibiotics

Antibiotic	Abbreviation	Final Concentration medium $\mu\text{g/ml}$
Sodium ampicillin	Ap	50
Chloramphenicol	Cm	50
Gentamycin sulphate	Gm	5
Kanamycin sulphate	Km	50/25
Nalidixic acid	Nal	100/50
Spectinomycin	Sp	50
Streptomycin sulphate	Sm	100
Tetracycline	Tc	10

vortexing or (b) adding an equal volume of late exponential culture to 2 x freezing medium (Section 2.12). In both cases storage was at -20°C . For short term storage colonies grown on plates were stored at 4°C in the dark for up to one month.

E. coli strains were maintained on NB agar containing appropriate antibiotics for up to one month at 4°C . For long term storage, strains were kept in stabs at room temperature, stab agar was NB plus 7g l^{-1} 'Bacto' agar. Gene banks or clones in E. coli were stored by mixing with 2 x freezing medium (Section 2.12).

2.7 Growth of organisms

2.7.1.1 Photoheterotrophic growth conditions - liquid cultures

Small scale cultures were grown in 100ml of PM contained in 250ml 19 Quickfit conical flasks. After inoculation (1% (v/v)) the flasks were capped with rubber suba-seals (William Freeman and Co Ltd, Barnsley, W Yorkshire) and flushed with O_2 -free N_2 for 15 min through inserted sterile syringe needles. Flasks were incubated in a shaking water bath at 30°C under a light intensity of $25\ \mu\text{Em}^{-2}\text{S}^{-1}$. Larger scale photosynthetic cultures were grown in 5, 10 or 20 l flat bottomed vessels (244/1350, Baird and Tatlock) with Quickfit tops which were sealed with suba-seals. After inoculation (0.2 - 0.4% (v/v)) the vessels were flushed with O_2 -free N_2 for 30 min and incubated at an incident light intensity of $35\ \mu\text{Em}^{-2}\text{S}^{-1}$ in a warm room at 30°C . All cultures were stirred continuously by magnetic stirrers. Growth was followed by the optical density of cultures at 540 or 650 nm in a Pye-unicorn SP500 Spectrophotometer.

2.7.1.2 Photoheterotrophic growth conditions - plates

Plates were incubated under anaerobic conditions in the light using the anaerobic bag technique described by Westmacott and Primrose (1975). Plates were placed on a tray within transparent nylon bags (Portex Ltd, Hythe, Kent) which were heat sealed and flushed with O_2 -free N_2 for 15 minutes. A beaker containing saturated pyrogallol solution (10ml) and 15% (w/v) potassium carbonate/10% (w/v) sodium hydroxide solution (15ml) was also placed in the bag to remove traces of oxygen. Incubation was at $30^\circ C$ at a high light intensity of $35 \mu Em^{-2} s^{-1}$.

2.7.2.1 Chemoheterotrophic growth conditions - liquid cultures

Rm. vanniellii was grown under microaerophilic conditions in darkness in 1L medical-flat bottles, containing 250ml of PMY medium, plugged with suba-seals and covered with foil to exclude light (or 4 bottles were placed in a 'tupperware' box covered with foil). The bottles were flushed with O_2 -free N_2 for 15 min and then the O_2 concentration was adjusted approximately by injection of either air or pure O_2 through a 0-2 μm millipore syringe filter and hypodermic needle. The cultures were incubated at $30^\circ C$ on a gyratory platform at 100 rpm. The atmosphere was maintained at a particular O_2 concentration by monitoring the headspace using a Pye gas chromatograph 104 series (model 34). A 500 μl sample of the gas was removed from the headspace and passed through a glass column packed with molecular sieve 5A (90-100 mesh) at $50^\circ C$ using high purity helium as a carrier gas with an inlet pressure of 30lb/sq inch and an outflow rate of 20ml/min.

2.7.2.2 Chemoheterotrophic growth conditions - plates

Colonies of Rm. vannielii were grown on plates incubated under microaerophilic conditions in darkness. The plates were placed inverted in a 'tupperware' box, the lid was placed on the box checking that the 'lip' was pressed firmly down all round and then the box was sealed by 'stretching' insulating tape round the box lid junction and then rubbing the tape to ensure that a good seal had been made. The box was then flushed with O₂-free N₂ through a hypodermic needle and holes made in the lid for 15 minutes. The holes were sealed with insulating tape and then the atmosphere was adjusted by injection of a known amount of air or oxygen to give the desired atmosphere. For incubation the box was placed in a 30°C incubator. The atmosphere in the box was monitored and adjusted as above.

2.7.3 Growth of E. coli

Liquid cultures of E. coli were grown at 37°C in 25ml universal tubes, static or shaken in Gallenkamp Orbital Shaker (150 rpm), depending upon the experiment. E. coli cells used for transformation were grown in 250ml flasks in a New Brunswick Gyrotory waterbath (275 rpm). Culture density was monitored at 550 or 600nm with an LKB Ultraspec 4050.

2.7.4 Culture harvesting

Unless otherwise stated, Rm. vannielii cultures were harvested in the late exponential phase of growth (OD 540 nm = 1.0-2.0) by centrifugation at 20,000 x g for 20 min at 4°C. When necessary cell pellets were stored at -20°C. Smaller volumes were harvested by centrifugation in an MSE

multex or MSE Chilspin at 5000 rpm for 10 min. During manipulations using Eppendorf tubes centrifugation was performed in an MSE microcentaur by a 2 min spin at high speed.

2.8 Enrichment for bacteriophage from the natural environment

Samples were obtained from a pond (near Leamington Spa, Warwickshire) and a sewage works settling tank (Finham, Coventry, West Midlands). Each sample was treated as follows: centrifugation was carried out in an MSE Multex (5000 rpm, 10 min). The supernatant was brought to pH 7.0 by addition of HCl or NaOH as appropriate, sterilized by shaking with a couple of drops of chloroform and then added to an equal volume of double strength PMY medium. The enrichment culture was placed in a 250ml B19 conical flask and inoculated with approximately 0.25×10^8 cells of Rm. vannielii Rm5 and the flask capped with a suba-seal and flushed with O_2 -free N_2 for 10 min.

The pellet obtained after centrifugation was resuspended in 1% (w/v) Beef extract, 50mM arginine pH 9.5 and shaken for 15 min; the mixture was recentrifuged as before, the supernatant was removed, brought to pH 7.0 and treated as above. This process was repeated with the pellet being resuspended in Beef extract/Arginine solutions with a series of pH values between 7 and 11.5. All of the resulting cultures were incubated in a shaking water bath under tungsten bulbs with a light intensity of $25 \mu E m^{-2} s^{-1}$. After 3-5 days growth, samples were removed from the cultures and sterilized either by filtration or chloroform treatment. Spots of each were placed on freshly seeded bacterial lawns in soft agar and a control spot of sterile PM was always placed on the same plate to check for dilution effects. The plates were incubated anaerobically in

the light using the gas bag technique (Section 2.7.1.2) until the lawns had fully developed and were then inspected for zones of inhibition or plaques.

2.9 Screening for interspecies bacteriocin or phage interactions

Two methods were used as follows:

(1) All available members of the Rhodospirillaceae were grown up in PMY medium in 25ml quickfit flasks sealed with suba-seals under anaerobiosis in a light intensity of $25 \mu\text{Em}^{-2}\text{S}^{-1}$ in a shaking water bath at 30°C . Cells (0.3ml) from late exponential phase cultures were used to 'seed' bacterial lawns (0.3ml of cells + 2.5ml of top agar) for each species/strain. Each culture was transferred to a 25ml universal tube and centrifuged in an MSE multex (5000 rpm for 10 min). The supernatants were transferred to fresh tubes and treated in two ways: (i) a sample was passed through a $0.2\mu\text{m}$ Millipore syringe filter (ii) a drop of chloroform was added to a sample of the supernatant which was then shaken vigorously. A drop of supernatant from each type of treatment for each species/strain was then placed on each of the prepared top agar lawns. The spots were allowed to dry and then the plates were incubated in anaerobic/light conditions using the gas-bag technique as detailed in Section 2.7.1.2. After 5 days the plates were inspected for signs of growth inhibition or plaques in the bacterial lawns in the 'spotted' areas.

(2) The cultures were grown up as above and then 20-50 μl of each culture were placed on fresh PMY plates and allowed to dry. The plates were then incubated as in Section 2.7.1.2 until there was a spot of

growth for each strain. Each plate was then inverted over ether for 5-10 min to kill the cells in each 'spot'. The plates were left to allow the ether to diffuse out of them before being overlaid with seeded bacterial lawns of each strain as above. The plates were incubated as above and then inspected for areas of growth inhibition around the 'colonies'.

2.10 Mitomycin-C induction of lysogens

Two methods were used as follows:

(1) Each species/strain was grown up in PMY to mid log phase and then the culture was divided into two aliquots (10ml in 25ml flasks covered with foil and stoppered with a cotton wool plug). To one aliquot Mitomycin-C was added to a final concentration of $10\mu\text{g ml}^{-1}$, the other aliquot being kept as a control. The cultures were then incubated further and the growth in each followed by monitoring the absorption at 260nm. When the control cultures had reached stationary phase, supernatants were prepared from all the cultures and 'spot-tested' on bacterial lawns as described in Section 2.9.

(2) Cells from log phase cultures of each strain were patched out on PMY agar and PMY agar containing Mitomycin-C at varying concentrations in the range $0.2-10\mu\text{g ml}^{-1}$. The plates were incubated aerobically/microaerobically in the dark. Any colonies that grew well on PMY agar but gave a mottled/lysed appearance or no growth at all, on the Mytomycin-C plates were tested further for Mytomycin sensitivity/lysogen induction as in above.

2.11 Preparation of synchronized swarmer cell populations

Described here is the standard procedure used in physiological studies and modifications of this method will be described where relevant.

Swarmer cells of Rm. vanniellii were prepared by passing a late exponential phase culture (A650 1.5-2) through a glass wool column in a similar fashion to that described by Whittenbury and Dow (1977). An 80cm long x 7.0cm wide glass column was packed with glass wool (BDH) to a depth of one third full, covered with aluminium foil and sterilized by dry heat at 106°C O/N. Before use, the column was washed with 2-4 litres of sterile distilled water. The culture was manually poured onto the top of the column; chains of cells and multicellular arrays were retained in the glass wool, while the swarmer cells passed through and were collected in a suitable foil covered vessel. The first 500ml of eluate was usually discarded. During filtration the column and collecting vessel were continuously gassed with O₂-free N₂. After collection the collecting vessel was sealed with a silicon bung with an inlet and outlet tube to facilitate gassing (of the headspace with nitrogen) which was carried out for 10 min. After gassing, the atmosphere could be kept constant by attaching a rubber bladder inflated with nitrogen to the inlet tube and clamping of the outlet tube so that a positive pressure was maintained within the vessel during sample removal. The swarmer cells were allowed to remain under these anaerobic dark conditions at 30°C for at least 15 min subsequent to synchronization to allow equilibration of the gas atmosphere, before the foil was removed and incubation continued at an incident light intensity of $35\mu\text{Em}^{-2}\text{s}^{-1}$ with magnetic stirring.

The homogeneity of the synchronized swarmer cell population was established by phase-contrast microscopy and cell volume distribution analysis. Routinely, between 5×10^7 and 2×10^8 swarmer cell ml^{-1} were obtained from the column as determined by Coulter counter analysis.

2.12 Cell volume distribution analyses/Coulter counter enumeration

A Coulter counter model 2BS and Coulter Channelyzer C1000 connected to a BBC microcomputer via local interface electronics was used to determine size distribution and cell count. Samples (50-200 μl) of the culture were diluted into 20ml of balanced salt electrolyte (Isoton; Coulter Electronics Ltd) and profiles were stored on floppy disc and printed on a Tandy TRS-80 plotter for comparison. The computer program used included a calibration file which compared cell-volumes with those of latex particle standards of known size distribution. Cell counts were taken as the mean of five determinations using the 30 μm orifice probe and an amplification setting of 0.5.

2.13 Nitrosoguanidine mutagenesis

Phototrophically grown cells were harvested and resuspended in fresh PMY broth at the desired density depending upon experiment. 50ml aliquots of the suspension were placed in 400ml medical flat bottles, sealed with suba-seals and gassed with O_2 -free N_2 for 10 min. The bottles were then placed on their sides on a rotating platform under tungsten lamps ($35 \mu\text{E m}^{-2}\text{s}^{-1}$) at 30°C to equilibrate. N-methyl-N-nitro-N-nitrosoguanidine (NTG) was added to one bottle to a final concentration of $100 \mu\text{g ml}^{-1}$ from a freshly prepared concentrated stock. 1ml samples were removed (using syringes and hypodermic needles) at intervals up to a maximum of

1 hr. Samples were taken from a control bottle, to which no NTG had been added, over the same period. Each sample was placed in an eppendorf tube, centrifuged in an MSE microcentaur (2 min at high speed), the supernatant was discarded and replaced with 1ml of PMY broth, the cells were resuspended and the process repeated twice further to effect complete washing of the cells which were finally transferred, in 1ml of PMY broth, to 9ml of PMY broth in pregassed 25ml quickfit flasks sealed with suba-seals. The cells were then left to express for a period of 6 hr or O/N before being diluted and plated out on PMYA plates and incubated as appropriate for the experiment.

2.14 Ultraviolet light mutagenesis

A mid exponential phase phototrophically grown culture was placed on ice and a 7ml sample was removed, pelleted in an MSE multex, resuspended in 7ml of phosphate buffered saline buffer and placed in a sterile glass petri dish with a magnetic flea over a stirrer and under a bacteriocidal lamp which had 'warmed up' for 30 min and was emitting short wave UV light. Irradiation was started by removing the glass lid of the petri dish and samples were removed at time intervals up to 180 seconds. The samples were either diluted immediately and plated out on PMY agar or pelleted, resuspended in PMY broth and incubated overnight before being diluted and plated out. In a control, cells were taken through the same manipulations except that they were not exposed to short wave UV light from the bacteriocidal lamp.

2.15 Preparation of cell-free extracts

Cell pellets were resuspended in ice-cold 10mM Tris-HCl buffer pH 7.4 to

a high cell density and disrupted either by two passages through a French Press at a pressure of 1010 lbs/inch² using a 5ml cell/3/8" diameter piston or by sonication. Sonication was executed using an MSE 12/76 Mk2 sonicator fitted with a microtip probe that could be used with eppendorf tubes. The cell suspension was kept cool in a methanol/ice bath and cells broken by 6 separate 15 second burst of ultrasound (20KHz: 6µm peak to peak amplitude) with 1 min cooling periods. Debris and unbroken cells were removed by centrifugation in an MSE HS21 centrifuge (30,000 x g 4°C 20 min) for volumes of extract greater than 10ml or using an eppendorf microfuge (4°C 10 min) for smaller volumes. The supernatants were removed and, if appropriate, stored at -20°C.

2.16 Transfer of plasmids to Rhodococcus vannieli by conjugation

In preliminary experiments, donor to recipient ratios, contact times and conditions, (dark/light;aerobic/anaerobic) were varied. No conclusions could be drawn from these experiments and a standard method was adopted as follows. The donor strain (usually E. coli HB101) was grown up in 10ml of NB plus appropriate antibiotics to select the plasmid. The culture was incubated static to a cell density of approximately 1×10^8 ml⁻¹. 1ml of this culture was then mixed gently with 1ml or 10ml of a late exponential phase Rm. vannieli culture and immediately filtered onto a 3cm diameter, 2µm pore size nitrocellulose filter using a Millipore vacuum filtration apparatus. Next 2 x 10ml of sterile PM broth were applied to the filter to wash any antibiotics away from the cells. The filter was then removed from the apparatus and placed on a PMY agar plate and incubated at 30°C O/N aerobically in the dark. The filter was then removed to a universal bottle containing 2ml of PMY broth, this was vortexed to remove the cells from the filter. The cell suspension

obtained was diluted and plated out on PMY plates with the appropriate selective and counterselective antibiotics and the plates were incubated at 30°C in dark microaerophilic conditions.

2.17 Measurement of enhanced auto-fluorescence

The method used was essentially that of Youvan et al (1983). The photographic apparatus used to measure enhanced IR fluorescence is diagrammed in Fig 2.17. A fluorescent light box was covered with a cardboard 'mask' with a hole cut out of it corresponding to the shape of the glass tray used to make the filter. The filter consisted of 500ml of 1.0M cupric sulphate in a 15cm x 10cm plastic box which was covered by a sheet of perspex on which the petri dishes to be photographed were placed. Plates were photographed with 35mm Kodak HIE 135 film through a 50mm Macro lens of f3.5 with a Hoya Infrared (R72) filter.

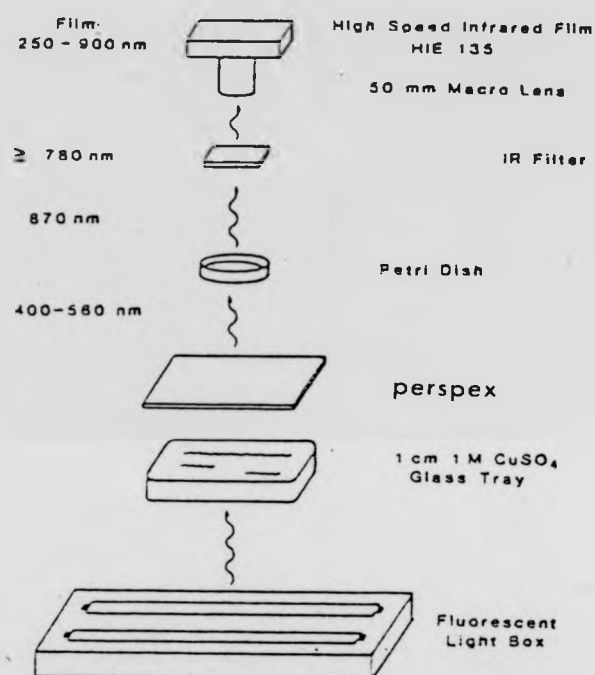


Figure 2.17 Photographic apparatus used to record near-IR fluorescence from bacterial colonies.

Petri dishes are irradiated with blue light, and photons emitted due to fluorescence with a wavelength between 780 and 900 nm from bacterial colonies are imaged by the camera and recorded by high-speed IR film. Components of the apparatus have been separated for display: in use the petri dish, perspex sheet and cupric sulphate filter are stacked. As drawn the apparatus is in fluorescence mode, and without the Hoya Infra red filter the bacterial colonies are photographed in absorption mode.

(adapted from Youvan *et al.* 1983)

2.18 Isolation of membrane fractions

Intracytoplasmic membranes were prepared from phototrophically and microaerophilically grown cells as follows. 3ml of cell free extract was layered onto a 33ml cushion of 25% (w/v) sucrose in 10mM Tris-HCl pH 7.4 and centrifuged (5h at 100,000 x g and 4°C) in the SW28 rotor of a Beckman L8 ultra-centrifuge. The soluble protein remained at the top of the cushion and the ICM pellet was resuspended in 10mM Tris buffer to a protein concentration of 15-25mg ml⁻¹ and stored as aliquots at -20°C. For smaller volumes of cell free extracts, 50-500µl of extract was layered onto a 4.3ml cushion of 25% (w/v) sucrose and centrifuged (2.5h at 100,000 x g and 4°C) in the SW50.1 rotor of a Beckman L8 ultra centrifuge.

2.19 Isolation of photosynthetic pigment-protein complexes

Triton-SDS gel electrophoresis

Membrane samples were solubilized at room temperature by mixing with an equal volume of 1.6% (w/v) SDS and 1.6% (w/v) Triton X-100 dissolved in 61mM Tris-HCl pH 6.8 buffer. The samples were then microfuged, the supernatant adjusted to 10% (w/v) glycerol and then run on the Triton-SDS semi-denaturing gel system described in 2.22.3.

2.20 Sucrose gradient centrifugation

Larger quantities of pigment-protein complex were prepared on sucrose gradients. Sucrose solutions (10, 20, 30 and 40% (w/v)) were prepared in 10mM Tris-HCl buffer pH 7.4 containing 0.05% (w/v) each of SDS and Triton X-100, sterilized by autoclaving (121°C, 10 min) and stored at

4°C. 10 to 40% discontinuous sucrose gradients were made in 5ml polyallomer tubes by layering on 1.1ml each of the above sucrose solutions. An equal volume of 68mM Tris-HCl pH 6.8 containing 1.6% (w/v) SDS and 1.6% (w/v) Triton X-100 was added to a membrane suspension containing 15-20 mg ml⁻¹ protein. After 5 min at room temperature, the mixture was microfuged (5 min) and the supernatant (300 - 500μl) layered onto the gradient.

After centrifugation to equilibrium (100,000 x g 16h, 4°C) in the SW50.1 rotor of a Beckman L8 ultra centrifuge, the gradients were fractionated (150μl aliquots) from the top. Sucrose concentrations were determined using a refractometer by reference to a calibration graph obtained using sucrose solutions of known concentration. Absorbance values were determined on a LKB Ultraspec Spectrophotometer.

2.21 Protein concentration determination

This was determined using the Folin-Phenol reagent (Lowry et al. 1951) with crystalline bovine serum albumin as a standard. Samples were diluted into 0.5ml distilled water in acid-washed boiling tubes and 0.5ml of 1M NaOH added. The tubes were boiled for 5 min and then cooled. To 50ml of 5% (w/v) sodium carbonate was added 0.5ml of 4% (w/v) sodium potassium tartrate and 0.5ml of 2% (w/v) copper (II) sulphate. After mixing, 2.5ml of this reagent was added to the samples and incubated for 10 min at room temperature. Folin-Giocalteu reagent (BDH) was diluted 1:1 with distilled water and 0.5ml added with vortexing to the sample tubes. After a further 30 min at room temperature, the absorbance at 750nm was measured against a reagent blank containing water. The protein content of the samples was determined by reference to a calibration

graph of absorbance against bovine serum albumen concentration.

2.22 Polyacrylamide gel electrophoresis (PAGE) of proteins

2.22.1 Completely denaturing 10-30% (w/v) polyacrylamide gradient gels

Gels were cast between large (20 x 25cm) glass plates, using 2mm thick teflon spacers, sealed by polypropylene tubing smeared with vaseline, and held together with bulldog clips and run in perspex electrophoresis tanks using a discontinuous buffer system. Alternatively the Biorad Protein II apparatus was used according to the manufacturer's instructions. Two solutions of 10% (w/v) and 30% (w/v) acrylamide were prepared from stock reagents as follows:

10% (w/v) acrylamide mixture	(50.0ml)
High bis acrylamide stock	8.3ml
distilled water	34.9ml
resolving gel buffer	6.25ml
10% (w/v) SDS	0.5ml

This mixture was degassed under vacuum in a dessicator (10 min) and immediately before pouring 10 μ l of N,N,N¹,N¹-tetramethylethylenediamine (TEMED) and 100 μ l of a freshly prepared 10% (w/v) ammonium persulphate solution were added to initiate polymerisation.

30% (w/v) acrylamide mixture	(20.0ml)
Low bis acrylamide stock	10.0ml
75% (w/v) glycerol	7.3ml
resolving gel buffer	2.5ml
10% (w/v) SDS	0.2ml

The mixture was degassed under vacuum in a dessicator (10 min) and immediately before pouring, 4 μ l TEMED and 50 μ l of 10% (w/v) ammonia persulphate solution were added. The 30% (w/v) mixture was placed in a magnetically stirred 25ml vial, stirred slowly and a suba-seal with inlet and outlet tubes inserted so that a few ml of the acrylamide was forced into the gel assembly flowing down the back plate. The 10% (w/v) mixture was pumped into the 30% acrylamide using a peristaltic pump at about a rate of 3ml/min with the stirrer set at maximum to ensure mixing. Thus the volume of liquid in the mixing vial remained constant, but was continuously diluted by the incoming lower percentage gel mix. The gradient was poured at room temperature and to ensure an even surface after polymerisation, the gel was carefully overlaid with a few ml of water saturated butanol and allowed to set for 1-2 hrs. After polymerisation the butanol was washed away with double distilled water and a stacking gel polymerized on top. The stacking gel mixture (10 ml) contained:

Stacking gel acrylamide stock	3.0ml
double distilled water	4.4ml
stacking gel buffer	2.4ml
10% (w/v) SDS	0.1ml

The mixture was degassed under vacuum for 10 min and immediately before pouring, 5 μ l TEMED and 100 μ l of 10% (w/v) ammonium persulphate added. Immediately after pouring into the gel assembly, a teflon gel comb was inserted into the stacker avoiding air bubbles. After the stacking gel had set (15 min) the gel comb was removed, the wells washed with distilled water, the polpropylene tubing and bulldog clips removed and

the gel placed in the gel tank ready for loading. Tris-glycine running buffer was prepared from stock solution as follows:

5 x Tris glycine reservoir buffer stock	300ml
10% (w/v) SDS	15ml
distilled water	1185ml

The upper and lower reservoir tanks were filled with running buffer and air bubbles removed from beneath the gel using a hypodermic syringe with bent needle. Protein samples containing known amounts of protein were routinely denatured in the presence of SDS and β mercaptoethanol using Laemmli sample buffer (Laemmli, 1970) as follows:

Stock Solutions

2 x		4x	
Stacking gel buffer	125 μ l	2 x stacking gel buffer	125 μ l
glycerol	100 μ l	glycerol	25 μ l
10% (w/v) SDS	200 μ l	20% (w/v) SDS	200 μ l
2 mercaptoethanol	50 μ l	2 mercaptoethanol	100 μ l
0.5% (w/v) bromophenol blue	25 μ l	0.5% (w/v) bromophenol blue	50 μ l

Complete denaturation was effected by boiling for 3-5 min before application to the gel using a Hamilton glass microsyringe (100 μ l capacity). In cases where a heat labile protein was involved denaturation by heating to 75°C for 2 min was found to be effective. 10-30% gradient gels were run in a 4°C cold room at 20mA constant current for about 16h in the case of the 'home made' system or with water

circulation in the BioRad Protean II system. Cooling tended to give better resolution than running at RT. The exclusion of the high percentage end of the gel was such that electrophoresis could be continued for 2-3h after the dye front had completely migrated off the end of the gel without protein loss. This was found to cause considerable zone sharpening of low molecular weight proteins. This was also attained by the inclusion of NaCl to a final concentration of 20mM in the gel and running buffer.

The stock solutions used in preparing 10-30% (w/v) gradient gels were as follows:

High bis acrylamide stock (acrylamide:bis-30:0.8)

acrylamide (Eastman Kodak)	60g
bisacrylamide (BioRad)	1.6g

The mixture was dissolved in 25ml double distilled water in a hot water bath and then made up to 100ml.

Low bisacrylamide stock (acrylamide:bis-30:0.15)

acrylamide	60g
bisacrylamide	0.3g

Dissolved as above

Both solutions were routinely filtered through Whatman qualitative filter paper before storage in the dark at room temperature.

Resolving gel buffer pH 8.8 (3M Tris-HCl)

Tris base	36.6g	pH accurately adjusted
Conc Hydrochloric acid <u>ca</u>	4.1ml	to 8.8 with HCl
distilled water to	100ml	

Stacking gel buffer pH 6.8 (0.5M Tris-HCl)

Tris base	5.98g	pH accurately adjusted
Conc Hydrochloric acid <u>ca</u>	4.1ml	to 6.8 with HCl
distilled water to	100ml	

Stacking gel acrylamide stock (acrylamide:bis-30:1.5)

acrylamide	10g
bisacrylamide	0.5g
distilled water to	100ml

5 x running buffer stock

Tris base	75.5g
glycine (BioRad)	360g
distilled water to	2.5l

2.22.2 Completely denaturing uniform
concentration gels

10% (w/v) acrylamide gels were cast using the high bisacrylamide stock (30:0.8) solution employed for gradient gels.

high bisacrylamide	12.5ml
distilled water	52.3ml
resolving gel buffer	9.4ml
10% (w/v) SDS	0.75ml

The mixture was degassed as before and polymerisation initiated by the addition of 15 μ l TEMED and 150 μ l 10% (w/v) ammonium persulphate. The solution was poured manually into the 'home made' gel plate assembly or the BioRad Protean II apparatus, overlaid with butanol and allowed to set. A 30% (w/v) acrylamide stacking gel of exactly the same composition as that used for gradient gels was then polymerized on top. Electrophoresis was carried out in a 4°C cold room either at 25mA constant current for 6h or at 15mA for 16h.

2.22.3 Incompletely denaturing uniform concentration gels

For the isolation of pigment protein complexes (Section 2.20.1) 7.5/10% (w/v) acrylamide gels were cast with a 3% (w/v) acrylamide stacker, both gels and stacker containing SDS and the non-ionic detergent Triton X-100 (BDH) both at a final of 0.05% (w/v). The running buffer also contained these detergents at the same concentration. The gels were electrophoresed at 25mA for 6h at 4°C.

2.22.4 Staining of Polyacrylamide gels Comassie blue staining

Gels were immersed in a solution of 45% (w/v) methanol, 10% (w/v) glacial acetic acid and 0.1% (w/v) Comassie blue R250 for 4-5h (500ml per gel) on a shaking platform. Destaining was effected by 3-4 successive soaks (1-2h each) in 45% (w/v) methanol, 10% (w/v) glacial acetic acid until the background was acceptable. Further destaining was done in 20% (v/v) propan-2-ol, 10% (v/v) glacial acetic acid

(overnight). This method was used when a lot of protein ($>100\mu\text{g}$ per track) was loaded on the gel.

Silver staining

The method of Wray et al (1981) was used as it is both relatively simple and quick to carry out. Gels were transferred to 50% (v/v) SLR methanol, made up in double distilled water, for at least 8h on a gently rotating platform. The methanol solution was changed 4-5 times during this period. The staining solution was prepared as follows:

Solution A : 1.6g Silver nitrate (Johnson Mathay Ltd) dissolved in double distilled water

Solution B : 42ml of 0.36% (w/v) sodium hydroxide containing 2.5ml of "0.880" ammonia solution (BDH)

Solution A was added dropwise to solution B with constant swirling and then made up to 200ml with double distilled water. The gel was transferred to the staining solution for 15 min with agitation and then washed in two changes of double distilled water for 5 min each. Prior to this washing, the gel was removed and placed on a glass plate, which had been previously cleaned with methanol, whilst the box was rinsed out with tap water and then deionized water before the gel was returned to the box. The protein bands were visualized by soaking the washed gel in developer solution, prepared by the addition of 2.5ml of 11% (w/v) citric acid and 0.4ml of 37% (v/v) formaldehyde to 500ml double distilled water. When the bands were sufficiently strong the staining process was stopped by transferring the gel to 45% (v/v) methanol, 10%

(v/v) acetic acid, in which the gels were stored.

2.22.5 Photography of Polyacrylamide gels

Gels were photographed on a light box using Panatomic X (32 ASA) 35mm monochrome film which was developed for 3 min in Kodak D19 developer and fixed in Kodafix for 5 min. A yellow filter (Pentax) was used to improve the contrast with Coomassie blue stained gels.

2.23 Extraction of chromosomal DNA from *Rm. vannielii*

Cell pellets were resuspended in TES buffer (pH 8.0) in 25ml universal tubes using about 10ml per 2g wet wt of cells. To this suspension was added $\frac{1}{2}$ volume of acetone. The mixture was vortexed and left at room temperature for 10 min. The cells were then repelleted in an MSE Multex or MSE Chilspin at 5000 rpm for 10 min. The supernatant was discarded and the cells washed by resuspension and pelleting in TES (pH 8.0) until the smell of acetone became negligible. The cell pellet was finally resuspended in TES (pH 8.0) using 10ml per g wet wt of cells and lysozyme was added to a final concentration of 1mg ml^{-1} and incubation carried out at 37°C for 60 min. After incubation sodium lauryl sulphate solution 25% (w/v) was added to give a final concentration of 2%. This normally brought about lysis upon mixing. A sample of the lysate was observed using phase contrast microscopy and if lysis was incomplete the cells were incubated at 60°C for 15 min. 5M NaClO₄ was added to the cell lysate to a final concentration of 1M to aid DNA-membrane separation. The mixture was then extracted with an equal volume of phenol saturated with TES. Following gentle mixing for 15 min, to form an emulsion the tubes were centrifuged in an MSE Chilspin (5000 rpm, 5 min, 4°C) and the

upper aqueous layer transferred to a fresh universal tube. The phenolic layer was easily recognised as it contained the photopigments. The aqueous layer was re-extracted with phenol, this time mixing for only 5 min. Next the aqueous phase was extracted twice using phenol:chloroform:isoamylalcohol (25:24:1) and finally extractions were performed using chloroform:isoamylalcohol (24:1) until no white precipitate remained at the aqueous-organic interface and the smell of phenol had been completely removed. Sodium chloride (5m) was added to the aqueous phase to a final concentration of 0.25m and then a double volume of ice cold ethanol added. The tubes were mixed by inversion and left until the DNA had precipitated (on ice 30 min/-20°C overnight) and it was then collected by centrifugation in an MSE Chilspin (8000 rpm, 10 min, 4°C). The pellet obtained was washed with 70% (v/v) ethanol (4°C) and left on ice for 10 min, recentrifuged, and dried under vacuum in a dessicator (30 min). The pellet was resuspended in TE buffer (pH 8.0 4ml per g original cell pellet) by leaving it agitating gently overnight at 4°C. If the pellet was resistant to going into solution it was heated to 65°C for 5-10 min. RNAase A (2mg/ml in TE, heat treated; Maniatis et al., 1982) was added to a final concentration of 100µg/ml and the solution incubated at 37°C for 30 min. The RNAase treatment was repeated and then sodium acetate (3m, pH 4.8) was added to a final concentration of 0.3m and the solution was extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1 and then with chloroform:isoamylalcohol until no white precipitate remained at the phase interface. Finally the aqueous phase was mixed with a double volume of 100% ethanol and left on ice for 30 min to precipitate the DNA which was then collected by centrifugation in an MSE Chilspin (8000 rpm, 10 min, 4°C). The DNA pellet was washed with 70% (v/v) ethanol, recentrifuged, dried under vacuum and resuspended in TE as above.

2.24 Isolation of plasmid DNA from E. coli

2.24.1 Large scale preparation

The method used was basically that of Clewell and Helinski (1970). E. coli cells were grown in 500ml of NB containing appropriate antibiotics for plasmid selection, to $2-4 \times 10^8$ cells/ml ($A_{600}=0.5$). If possible, amplification of the plasmid was carried out by adding spectinomycin to a final concentration of $300\mu\text{g ml}^{-1}$ and incubation extended for a further 18h. Cells were harvested in an MSE HiSpin 21 centrifuge using a 6 x 300ml rotor (10,000 rpm, 10 min, 4°C) resuspended in 16.5ml Tris-sucrose solution (0.05m Tris-HCl, pH 8.0, 25% (w/v) sucrose) and transferred to siliconized 250ml bottles which were placed on ice for 5 min, 4.5ml EDTA (0.25m, pH 8.0) was added and the solution was kept on ice for a further 5 min. Cells were lysed by the addition of 18ml of lysis mix (0.05 Tris-HCl, 0.0625m EDTA, 2% Brij 58, 0.4% sodium deoxycholate, pH 8.0) and the mixture was inverted until it appeared clear. Incubation at 42°C for a few minutes aided this process. The lysed solution was transferred to 50ml polycarbonate 'Oakridge' tubes and the unlysed cells, all debris, and intact chromosomal DNA was pelleted in an MSE HiSpin centrifuge using a 8 x 50ml rotor (18,000 rpm, 20 min, 4°C). The supernatant ("cleared lysate") was carefully transferred to a fresh tube, and was used to make 3 x 10ml CsCl gradients, or was frozen at -20°C until needed. For a 10ml gradient, 7.76ml cleared lysate, 7.38g CsCl and 0.27ml ethidium bromide (2.5% w/v) were gently mixed and kept at room temperature until all the CsCl had dissolved. The R_f of the resulting solution was ca 1.393 and this was poured into 10ml Beckman polyallomer tubes and the remaining space was filled with liquid paraffin. Gradients were centrifuged in a Beckman L8 ultracentrifuge using a 70Ti rotor

(36,000 rpm, 60h, 15°C). The plasmid DNA observed upon illumination of the tubes with long wave UV light, was removed through the side of the tube using a size 21 hypodermic needle and a 2ml syringe. The DNA was extracted thrice by vigorous shaking with an equal volume of propan-2-ol equilibrated with CsCl and TE (80ml propan-2-ol, 20g CsCl, 20ml TE) followed by centrifugation in an MSE microcentaur (1 min high speed). The upper layer, containing ethidium bromide was discarded. The DNA solution was dialysed against 5L of TE for 4h and then O/N against 5L of fresh TE.

2.24.2 Rapid small scale preparation

This was similar to that described by Maniatis et al (1982). Cultures were grown overnight in 5ml NB with appropriate antibiotic selection. Cells were harvested in an MSE Multex centrifuge (5000 rpm, 10 min) resuspended in phage buffer, and transferred to 1.5ml Eppendorf tubes. All subsequent centrifugation steps were carried out in an MSE Microcentaur at high speed, at room temperature. After centrifugation (1 min), the supernatant was removed and the cells were resuspended in the drop of residual liquid in the tube. Ice-cold solution I (150µl of 50mM glucose, 10mM EDTA, 25mM Tris HCl, pH 8.0) was added, mixed by gentle vortexing, and stored at room temperature for 5 min. Then 200µl of solution II (0.2M NaOH, 1% SDS; stable for 2 weeks at 4°C) was added, and the tubes inverted rapidly three times and stored on ice for 5 min. Tubes were inspected, and the contents mixed by inversion as necessary to obtain an almost clear solution. Ice-cold potassium acetate (150µl, pH 5.0; made by mixing 60ml 5M potassium acetate with 11.5ml glacial acetic acid and 28.5ml of water) was added, and the tubes agitated immediately by three cycles of rapid inversion and momentary vortexing.

Following at least 5 min storage on ice the tubes were centrifuged (5 min) to remove unlysed cells, membrane bound chromosomal DNA and debris and the supernatant transferred to a fresh tube using a Gilson micropipetter. An equal volume (ca 400 μ l) of phenol/CHCl₃ was added and the tubes vortexed for 5 sec and the layers separated by centrifugation (1 min). The upper layer was transferred to a fresh tube containing 400 μ l CHCl₃/isoamylalcohol (24:1), vortexed for 5 sec and centrifuged again (1 min). The upper layer was removed to a fresh tube, taking care to leave behind any white protein precipitate at the interface, and to this 2 volumes of 100% ethanol were added.

2.25 Restriction endonuclease digestion

The low, medium and high salt buffers were prepared at 10 x concentration and used as described by Maniatis et al (1982). BRL core buffer was used for enzymes Hin III and Pst I. After adding 1 μ l of 10 x restriction buffer for every 9 μ l DNA restriction enzyme was added and the digestion carried out at 37°C for at least 90 min.

2.26 Ligation

Restricted DNAs were mixed in appropriate volumes of TE buffer. To clone fragments a 4:1 fragment:vector ratio was used with a DNA concentration >50 μ g/ml. To promote recircularization, the DNA concentration was reduced to 10 μ g/ml. The mixture was heated to 65°C for 5 min, and allowed to reanneal slowly on ice for 1h. After adding appropriate amounts of 10 x ligation buffer (4mM ATP, 66mM MgCl₂, 0.1m DTT, 0.66M Tris-HCl, pH 7.06) and T4 ligase, the mixture was incubated at 15°C for at least 18h.

2.27 Concentration and purification of DNA

2.27.1 Extraction of DNA with phenol/chloroform

Phenol/chloroform mix was prepared by dissolving 100g of phenol and 100mg of 8-hydroxyquinoline in 100ml of chloroform and 4ml of isoamylalcohol. This was equilibrated by shaking the mixture with two changes of 0.2 volume 1M Tris (pH 8.0) and two changes of 0.2 volume TE, before storing at 4°C. using gentle shaking, DNA samples were mixed with an equal volume of phenol/chloroform until an emulsion formed. The two phases were separated by centrifugation in an MSE microcentaur (1 min, high speed). The upper aqueous phase was recovered, taking care not to disturb the interface, and the extraction process was repeated. A further extraction with chloroform/isoamylalcohol (24:1) was carried out as above to remove any traces of phenol. To maximise recovery, the organic or phenolic phase was mixed with an equal volume of TE, mixed and centrifuged (1 min, high speed). The aqueous phase was then extracted with chloroform/isoamylalcohol and pooled. DNA was recovered by ethanol precipitation.

2.27.2 Ethanol precipitation of DNA

To a DNA solution, half a volume of ammonium acetate (7.5M, pH 7.5, Sigma # C-7262) and three volumes of 100% ethanol (-20°C) were added, mixed by vortexing and chilled at -20°C overnight. The DNA was removed by centrifugation in an MSE microcentaur (10 min, high speed, room temperature). The supernatant was discarded and residual ethanol was removed from tube walls. The DNA pellet was dried under vacuum and resuspended in TE buffer.

2.28 Agarose gel electrophoresis

Horizontal agarose slabs were prepared by boiling agarose (Sigma type I, # A6013) in TAE electrophoresis buffer, 6% (w/v) gels were routinely used. The agarose was cooled to ca 50°C before addition of ethidium bromide (10mg/ml soln) to a final concentration of 0.5µg/ml and pouring. DNA samples were prepared by adding 0.1 volume of loading buffer and loaded into the gel slots. Electrophoresis was carried out with the gels completely immersed in electrophoresis buffer at 80-100 volts, or 25 volts when left to run overnight. DNA was visualized by transillumination with short wavelength UV light (200 nm) and photographed using Poloroid type 665 film. Restriction fragments were 'sized' using the 'DNA size' program (G Russell and N Crickmore) adapted from Schaffer and Sederoff (1981) and run on a BBC model B microcomputer.

2.29 Transfer of DNA from agarose gels to nitrocellulose filters

The method used was a simplified version of the original methods described by Southern (1975). The gel was stained and photographed as described above and then the portion of the gel to be transferred was cut out and one corner of the gel cut off as an orientation mark. The gel was immersed in a denaturing solution, (0.5M NaOH, 1.5M NaCl) for 40 min the solution being replaced with a fresh one after 20 min. The gel was rinsed in double distilled water and transferred to a neutralizing solution (50M Tris-HCl, pH 7.4, 1.5M NaCl) again for 40 min with a change of solution after 20 min. The gel was then placed upside down on a sheet of blotting paper soaked in 20 x SSC (3M NaCl, 0.3M Sodium citrate) with its ends resting in a reservoir of 20 x SSC. A piece of

nitrocellulose filter (Millipore Cat No HAHY0010, 0.45 μ m pore size) was cut to match exactly the shape and dimensions of the gel. The nitrocellulose was wetted with 2 x SSC and laid carefully on the surface of the gel ensuring that it did not overhang the edges and that no air bubbles were trapped between the gel and the nitrocellulose. A piece of filter paper (Whatman 3mm paper) of the same dimensions as the gel and nitrocellulose was wetted with 2 x SSC and placed carefully on the nitrocellulose, again making sure that there were no air bubbles trapped between them. Then a 3cm stack of filter papers of the same dimensions were placed on top followed by a stack of tissues of roughly the same size. A glass plate was placed on top and the whole assembly wrapped in cling film and a weight placed on top. Transfer was allowed to continue overnight. The assembly was dismantled and the filter rinsed in 2 x SSC, blotted dry and baked in a vacuum oven at 80°C for 90 min. The filter was then stored under vacuum at room temperature if not being used for hybridization.

2.30 Oligonucleotide labelling of DNA

The method used was a simplification of that described by Feinberg and Vogelstein (1982) in which random sequence hexadeoxynucleotides hybridize to the restriction fragment of interest and, in the presence of radioactive deoxynucleotide triphosphates and the 'Klenow fragment' of DNA polymerase I prime the synthesis of radiolabelled copies of the fragment. The fragment to be labelled was prepared as detailed in Section 2.31. The amount to be labelled (up to 25 μ g per labelling reaction) was heated to 100°C for 5 min and stored at 37°C for at least 10 min prior to use. The labelling reaction was carried out at room temperature by addition of the following reagents in the stated order in

an Eppendorf tube: $x\mu\text{l}$ H_2O (to give a final total volume of $25\mu\text{l}$); $5\mu\text{l}$ of OLB buffer prepared as described in Section 2.4; $1\mu\text{l}$ of Bovine Serum Albumin (enzyme grade BRL 5561 $10\text{mg}/\text{ml}$); $x\mu\text{l}$ DNA fragment (25ng), up to $16.25\mu\text{l}$; 2.5 ^{32}P α -dCTP ($10\mu\text{Ci}/\mu\text{l}$) (Amersham International); $0.5\mu\text{l}$ Klenow (large fragment/Polymerase I, 2 units). The reaction was allowed to proceed for at least 5h; (usually overnight) and $75\mu\text{l}$ of TE pH 8.0 was added before storage at -20°C or use in hybridization. The whole reaction mixture was used in hybridization and this did not produce any undesirable background or non-specific binding to the filter so the step of separating unincorporated nucleotides was not carried out.

2.31 Preparation of DNA fragments from agarose gels

The procedure published by Dretzen *et al* (1981) was used. DNA fragments were separated by electrophoresis in 0.5% to 1.5% (w/v) agarose gels (Section 2.29). DNA bands were visualized with long wave UV light only. The gel was rotated through 90° , slits made at one end of the DNA bands required, a piece of DEAE cellulose (Whatman, DE81 paper, prewashed in 2.5M NaCl overnight, washed several times in water and stored in 1mM EDTA at 4°C) placed into each slit, and the gel squeezed firmly against the papers to close the incision. TAE buffer was added to the tank until it barely covered the surface of the gel. Electrophoresis was resumed until the DNA had entered the DEAE-cellulose paper strips. The DEAE-cellulose paper strips were blotted dry, placed in 1.5ml Eppendorf tubes and $400\mu\text{l}$ of 20mM Tris-HCl (pH 7.5), 1mM EDTA, 1.5M NaCl was added. The papers were shredded by vortexing and incubated in foil wrapped tubes at 37°C for 2h to elute the DNA. The mixture was transferred to Eppendorf tubes containing a plug of siliconized glass wool (to trap the paper shreds). A small hole was made in the bottom of

each tube, which was placed on the tip of another Eppendorf tube which in turn was placed in a universal bottle. After centrifugation for 1 min at 4000 rpm, the eluate was extracted with 3 volumes of water saturated butan-1-ol and the DNA was precipitated by adding 2 volumes of ethanol (Section 2.27).

2.32 Hybridization to Southern filters

The method used was a simplified version of that described by Maniatis et al (1982). The baked filter was removed from between the sheets of filter paper and floated on and then immersed in 6 x SSC for 1-2 min with agitation. The filter was then placed between two plastic sheets and sealed using a heat sealer (Calor Ltd) on three sides to form a bag. To this was added 2-3 ml of preheated (68°C) pre-hybridization fluid, (6 x SSC; 5 x Denhardt's solution (from 50 x Denhardt's prepared as described by Maniatis), 0.5% (w/v) SDS; and 100 µg/ml salmon sperm DNA (from 10 mg/ml stock, heated to 100°C for 5 min and cooled on ice for 5 min before addition); all air bubbles were excluded from the bag before it was sealed. The filter was left to pre-hybridize for at least 2h or overnight, at 68°C in a waterbath with constant agitation. The bag was cut open and the filter transferred to a fresh bag and the hybridization fluid was added using just enough to keep the filter wet (50 µl/cm² of filter). The hybridization solution was 6 x SSC, 0.01M EDTA; 5 x Denhardt's solution; 0.5% SDS; ³²P-labelled denatured probe DNA (prepared as in Section 2.31); 100 µg/ml denatured salmon sperm DNA. The latter two were mixed together and boiled for 5 min, left on ice for 5 min before being added to the hybridization mix. Air bubbles were excluded from the bag which was then heatsealed and left incubating at 68°C overnight. The filter was removed from the bag and immediately immersed in 100 ml of 2 x

SSC; 0.5% (w/v) SDS and allowed to wash for 10 min with agitation. This was repeated with fresh 100ml of washing solution. The filter was then washed for two 30 min periods at 65°C in 0.1 x SSC; 0.1% SDS using 100ml each time. Then the filter was inspected using a Geiger counter and if the background was still too high (>4 cps) the filter was subjected to a further wash at 65°C for 30 min. The filter was then blotted dry between two pieces of Whatman 3mm filter paper and sealed in a bag for autoradiography.

2.33 Autoradiography

This was carried out in a similar fashion to that described by Maniatis et al (1982) using x-ray film and Dupont intensifying screens.

2.34 Transformation of E. coli

Transformation with plasmid DNA was carried out as described by Maniatis et al (1982). During the construction of the gene library and the cloning of Tn₅ containing fragment certain modifications were employed. An overnight culture of HB101/DH1 was subcultured (1:50 or A₅₅₀ = 0.05) and grown up in 50ml SOB medium, in a 250ml flask (275 rpm, 37°C) to A₅₅₀ = 0.35. Cells were then treated as in Maniatis et al (1982) and all steps were carried out in a 4°C cold room using chilled pipettes and eppendorfs. Centrifugation steps were performed gently using an MSE Chilsplin (3000 rpm for 10 min). Following heatshock cells were incubated in 10ml SOB for 90 min at 37°C to allow expression of antibiotic resistance.

CHAPTER 3

ATTEMPTS TO FIND AN INDIGENOUS VECTOR WHICH COULD BE
EXPLOITED IN THE DEVELOPMENT OF A GENE TRANSFER SYSTEM

3.1 Introduction

In developing a system of genetic analysis for an organism such as Rm. vanniellii in which no previous system for in vivo genetic manipulation was available, the strategy was to try a wide variety of approaches in the hope of finding one that worked. Initially it was decided to search for an indigenous vector, either phage or plasmid, which could be exploited for gene transfer. Such a replicon would have advantages over other 'foreign' entities in that possible problems due to non-replication would be avoided; the indigenous replicon would have promoter sequences which would be recognized by Rm. vanniellii, allowing expression of inserted DNA sequences, whereas promoter sequences from extraneous plasmids might be recognized less efficiently or not at all. Additionally such a replicon would be less likely to be degraded by the host's restriction enzymes.

Both lysogeny and bacteriocinogeny are found amongst members of the Rhodospirillaceae. These phenomena are important for several reasons; temperate phage and bacteriocins may be encoded either by chromosomal or extrachromosomal elements and are therefore of considerable intrinsic genetic interest. Temperate phage can be exploited as vectors of chromosomal DNA during transduction and plasmids which code for bacteriocins could be developed as cloning vehicles.

Virulent phage have been isolated for several purple non-sulphur bacteria, namely Rhodospseudomonas palustris (Freund-Mölbart et al. 1968; Boseker et al. 1972a,b), Rhodobacter capsulatus (Schmidt et al. 1974), Rb. sphaeroides (Abellovich and Kaplan, 1974; Donohue et al. 1985) and

Rhodopseudomonas blastica (Eckersley and Dow, 1980). A number of temperate phage have been isolated for Rb. sphaeroides using mitomycin C induction (Mural and Friedman, 1974; Pemberton and Tucker, 1977; Tucker and Pemberton, 1978; Tucker and Pemberton, 1980; Duchrow et al., 1985; Duchrow and Giffhorn, 1987). Pemberton et al. (1983) found that all of seventeen natural isolates of Rb. sphaeroides contained between one and three prophage. In contrast, all the 95 phage isolated for Rb. capsulatus (Wall et al., 1975) were virulent. To date no phage capable of transduction has been isolated for any member of the Rhodospirillaceae. However a gene transfer agent (GTA) of Rb. capsulatus (Solioz et al., 1975) does mediate a form of generalized transduction. The GTA is a non-infective, small phage-like particle.

Bacteriocins are of interest because they are often encoded by plasmids, and as such could be very useful for the development of a gene transfer system. Bacteriocins which are active against strains of the same or related species are produced by a wide variety of Gram-negative and Gram-positive bacteria. The occurrence of this phenomenon amongst the photosynthetic bacteria was first reported by Guest (1974). During an unsuccessful attempt to find either lysogenic strains or phage active against members of the Rhodospirillaceae, specific inhibitory interactions resembling bacteriocinogeny were observed in certain strains of Rb. capsulatus, Rb. palustris and Rb. sphaeroides. Wall et al. (1975) confirmed the presence of bacteriocins in 9 out of 33 wild-type strains of Rb. capsulatus. Thus the capacity to produce bacteriocins is evidently common amongst wild-type strains of Rb. capsulatus.

3.2 Attempt to detect cross-reactions between members of the Rhodospirillaceae which might indicate phage or bacteriocin production

3.2.1 Results

Two methods were used as described in Section 2.9. In the first method 18 different strains were used and cell-free supernatants from photosynthetically-grown liquid cultures were spotted onto seeded bacterial lawns, which were then incubated, in gas-bags, under photosynthetic conditions. No sign of inhibitory interactions were observed either with chloroform-treated or filtered supernatants. In the second more sensitive method, 21 strains were tested (the 20 strains listed in Table 2.1.1b plus Rm. vannieli Rm5). 200 μ l samples from photosynthetically-grown liquid cultures were spotted onto agar plates which were incubated under photosynthetic conditions to give spots of growth. The plates were then treated with either vapour to 'kill' the cells before being overlaid with lawns of the 21 strains. The results confirmed those of the first method, with none of the 18 strains showing any inhibitory interactions (ie no inhibition of lawn growth around the dense colonies). However one of the three additional strains Rs. rubrum WRR showed inhibition of growth against 16 of the 21 strains tested. The four strains that were not inhibited were Rhodopseudomonas acidophila ATCC 25092 and three strains of Rm. vannieli: RV2, RV21A and RV1. The 1-2mm zones of inhibition were observed around each spot of Rs. rubrum WRR and interestingly it also exhibited autoinhibition. When filtered or chloroform-treated supernatants from cultures of this strain were plated with cultures of sensitive strains no plaque forming activity could be detected. This suggested that the inhibitory interactions were due to bacteriocinogeny rather than phage production.

3.2.2 Discussion

Guest (1974) found that Rs. rubrum NCIB 8255 neither inhibited nor was inhibited by any other of the strains tested, and in this study the same strain showed no inhibitory effects. Guest found that within the 6 strains of Rb. sphaeroides used, intra-specific inhibitory reactions were few and weak but all the Rb. sphaeroides strains inhibited at least one strain of Rb. capsulatus. In particular Rb. sphaeroides NCIB 2253 showed inhibition of two strains of Rb. capsulatus. In this study, 2253 did not inhibit either of the two strains of Rb. capsulatus used. Guest found that the Rb. capsulatus group showed the greatest amount of inhibitory activity. 8 out of 12 strains used were very active against most of the 6 strains of Rb. sphaeroides, and against the other 4 strains of Rb. capsulatus. The strain of Rb. capsulatus used here showed no intra or inter-specific inhibitory activity. Guest used 8 strains of R. palustris and found very limited intra-specific interaction and only half of them were significantly active against Rb. capsulatus indicator strains. The strain of R. palustris used here showed no inhibitory interactions under the conditions used.

The most important observation in this study was that none of the Rm. vanniellii strains used showed any inter or intra-specific inhibitory activity. The positive result with the Rs. rubrum strain suggested that the methodology was correct, and consequently this type of investigation was pursued no further. However it is surprising that only one of all the strains tested showed any inhibitory activity. No controls using strains known to have inhibitory activity were included. It is possible that the 'Warwick' strains have lost such activities during continual sub-culturing over the years.

3.3 Attempt to detect prophage in members of the Rhodospirillaceae by induction with mitomycin C

It is known that phage formation can be induced in lysogenic bacteria such as *E. coli* K-12 by mitomycin C (Otsuji *et al.* 1959) and this method has been used to isolate temperate phage from *Rb. sphaeroides* (eg Mural and Friedman, 1974). An initial experiment showed that 12 out of 18 strains of Rhodospirillaceae tested were inhibited in growth by mitomycin C at $10\mu\text{g ml}^{-1}$. Cultures were grown in 25ml flasks stoppered with cotton wool bungs in the dark. Under these conditions the *Rm. vannieli* strains exhibited poor growth with clumping. At this time the microaerophilic growth technique (Chapter 4) had not been developed and so there was no way of obtaining reproducible growth in the dark. Growth in the dark is essential for experiments involving mitomycin C, since it is degraded by light. Because of this problem, this approach was abandoned. The sensitivity to mitomycin C observed in the preliminary experiment may be due to the lysogenic nature of *Rm. vannieli*, and this merits further investigation using the new microaerophilic growth techniques. The procedures for detecting lysogeny using mitomycin C are given in Section 2.10. In addition it would be interesting to use UV light for induction of putative lysogenic phage.

3.4 Attempted enrichment for bacteriophages from samples from the natural environment

Samples were obtained from two different anaerobic environments in which purple non-sulphur bacteria were likely to be found: mud from the bottom of a pond near Royal Leamington Spa, Warwickshire; sediment from a sewage works settling tank, Finham, Coventry. The samples were initially used to carry out an enrichment as described in 2.8. This method

included procedures designed to release phage particles from the association with other solid material. As a result of this attempt no plaque-forming activity was detected using Rm5 for enrichment, and in bacterial lawns. A simpler method in which samples from the same sources were treated with chloroform and added to mid-log phase cultures of the nalidixic acid resistant strain of Rm5 (RB1) was performed. The cultures were incubated phototrophically for a further two days, treated with chloroform and centrifuged to remove the cells. The supernatants were plated with phototrophically-grown cells, and incubated phototrophically until lawns had developed. Again no plaque-forming activity was observed. No positive controls, i.e. demonstration of isolation of phage for enteric bacteria which are more common in such environments, were used to show that the methodology was working. Neither was it shown that Rhodomicrobium strains were actually present in the environments sampled.

3.5 Discussion

No phage or bacteriocin type activity was detected amongst any of the Rm. vannielii strains tested here. It would be interesting to repeat the tests using the microaerophilic growth technique (Chapter 4) since the production of inhibitory activities may be growth-mode specific. The mitomycin C-induction experiments should be carried out as described in Section 2.10 using microaerophilic/dark conditions.

Unfortunately no phage for Rm. vannielii has yet been found. To find a phage and one capable of transduction would be doubly fortunate since no phage capable of transduction has been isolated for any member of the Rhodospirillaceae.

As described above, several of the experiments could not be carried out satisfactorily due to the lack of an alternative growth mode to photosynthesis.

CHAPTER 4

GROWTH CONDITIONS FOR RHODOMICROBIUM VANIELII

ON/IN SOLID MEDIA

4.1 The 'gas-bag' incubation technique

4.1.1 History of the use of the 'gas-bag' technique for growth of purple non-sulphur bacteria

The 'gas-bag' technique (Westmacott and Primrose, 1975) whereby the anaerobic culture of members of the Rhodospirillaceae in illuminated petri dishes is achieved, was used routinely in this establishment for the purification of cultures prior to this project. The method used was cheap and simple and had advantages over other systems which had previously been tried. Traditionally, purple non-sulphur bacteria were isolated from the environment, with or without an initial enrichment step, as colonies grown photoheterotrophically from an inoculum which had been diluted and suspended in molten agar containing nutrients. This method did not facilitate easy subculturing as the colonies were embedded in agar and the organisms often grew in a film between the agar and the glass. Skerman (1967) described a method of sealing petri dishes, together with a small amount of alkaline pyrogallol to absorb traces of oxygen, onto a glass sheet. However, this was not practical if a large number of plates were required.

Photoheterotrophs can be grown in petri dishes standing in glass McIntosh and Fildes or Brewer Jars (Swoager and Lindstrom, 1971), but this has the disadvantage of limited capacity in the containers and the inability to illuminate each plate uniformly. The use of polycarbonate anaerobic jars was found to be unsatisfactory in that they became saturated with moisture (Collee *et al.* 1972; Westmacott and Primrose, 1975), and this problem was verified in this study. The addition of silica gel crystals to the system did not cure this problem, as they

rapidly became saturated.

A couple of continuously-gassed systems have been used. The first, (Sistrom, 1966) involved a lucite box, with a capacity of 12 petri dishes, which was gassed continuously during incubation in an illuminated water bath. In another system, Hill (1973) described the use of a continuously-gassed bag. Both of these methods required large amounts of oxygen-free gas, as the time taken for colonies to appear is generally at least two days.

The method described by Westmacott and Primrose (1975), achieved good photosynthetic growth of Rm. vanielii, Rhodopseudomonas acidophila and Rhodopseudomonas palustris in times considerably less than those required for aerobic growth (eg 4 days vs. 12 days for R. palustris). Several advantages were cited for the use of anaerobic bags for incubation of the Rhodospirillaceae: it was cheap, did not waste space, did not involve explosive gases or high vacuum and produced uniformly pigmented colonies of the organism in a very short time, relative to aerobic cultures. It had a number of advantages over the conventional "agar-deep" method in that selected colonies could be easily picked for subculture. The method currently used for photoheterotrophic growth of members of the Rhodospirillaceae at Warwick is basically that of Westmacott and Primrose, and is described in Section 2.7.1.2.

4.1.2 Attempted isolation of spontaneous antibiotic resistant mutants using the 'gas-bag' incubation technique

4.1.2.1 Introduction

Antibiotic-resistant mutants are essential in microbial genetics for such things as strain verification, counter-selection in conjugation experiments and as selectable markers in chromosome mapping.

Spontaneous antibiotic resistant mutants are generally easily selected by spreading a suspension containing 10^8 - 10^9 cells on an agar plate containing a suitable growth medium and the antibiotic. Only those cells which are resistant will multiply and form visible colonies.

4.1.2.2 Results

Several attempts were made to obtain antibiotic resistant mutants of Rm. vanniellii using the 'gas-bag' incubation technique. Cells were grown up in liquid cultures under photoheterotrophic growth conditions, harvested and resuspended in buffer. Aliquots containing between 5×10^8 and 5×10^9 cells were spread on PYM agar plates containing either Streptomycin, Ampicillin, Nalidixic acid or Rifampicin at concentrations of 10, 50 and $100 \mu\text{g ml}^{-1}$. After five days incubation, very small colonies were observed on some of the plates. These were restreaked on fresh plates containing antibiotics at the same concentration, but in all cases, no regrowth occurred. After several attempts, using increasing numbers of cells spread on the plates, no antibiotic-resistant mutants had been isolated and this approach was abandoned.

Various problems were encountered using this method, apart from the fact that it did not yield any antibiotic resistant mutants. A thermometer placed on the tray inside the gas-bag recorded temperatures of 35-37°C whilst under the incubation conditions usually used, ie under illumination from tungsten bulbs in a 30°C warm room with a fan circulating air around the outside of the bag. 37°C is the upper limit for growth of Rm. vannielii and cells grown at this temperature show aberrant morphology.

In addition, during the long period taken for colonies to appear, the medium became severely dehydrated and, at the end of incubation, a pool of condensed water was found in the lid of each petri dish.

Irritatingly, the agar surface was frequently soaked with this condensate, especially during removal of the bags from the warm room or in the opening of the bag to inspect the plates. An interesting observation was that on some plates where the condensate came into contact with the agar surface during incubation, colonies grew well at the water-agar interface, but failed to grow (or grew very poorly) on the rest of the plate.

4.1.2.3 Discussion

These problems are caused by the system used for illumination. The major absorption maxima of the photo-system of the purple non-sulphur bacteria lie in the infra-red region and thus tungsten lamps are used as a source of light. Infra-red radiation has a heating effect causing a rise in temperature of the agar and the microenvironment of the sealed 'gas-bag'. Over the long incubation period this causes dehydration of the plates and the concentration of the components of the medium. When

combined with decreased water availability, and temperatures up to 37°C, such variability is likely to result in restricted growth.

4.2 The agar-tube incubation technique

4.2.1 History of the agar-tube technique

In 1907 Molisch succeeded in isolating some purple non-sulphur bacteria in pure culture from single colonies in agar tubes (Kondrateva, 1965). As a result of extensive experimental work, van Neil (1944) concluded that purple non-sulphur bacteria could be conveniently grown by the same method as purple and green sulphur bacteria. van Neil (1931) described the isolation of members of the former group using an agar tube method. Enriched cultures, developed from samples of silt or water from polluted sources, were used as inocula for dilution series in liquified agar tubes. Using this method, van Neil was able to isolate purple sulphur bacteria from a large variety of natural sources, in a simple mineral medium containing hydrogen sulphide in the form of Na_2S . When incubated in the light, colonies appeared in the agar within four to seven days. Tubes containing isolated colonies were cut open and the agar column transferred into sterile petri dishes when the colonies were removed and subcultured. Pure cultures were obtained after repeated passage from isolated colonies. A similar method is routinely used for the isolation of oxygen-sensitive anaerobes and is known as dilution-shake culture (Stanier *et al.*, 1976).

Table 4.2.2 Determination of spontaneous antibiotic-resistance frequencies using the agar tube technique

Antibiotic ($\mu\text{g ml}^{-1}$)	Frequency of spontaneous resistance
Nal 50	4×10^{-10}
Tc 15	$< 5.7 \times 10^{-10}$
Ap 50	$< 5.7 \times 10^{-10}$
Sp 100	5.8×10^{-10}
Km 25	$< 5.7 \times 10^{-10}$

Frequencies were calculated from the number of colonies developing in an agar column containing the antibiotic and seeded with a known number of colony forming units (cfu). The strain used was Pm vanielii R32.

4.2.2 Isolation of antibiotic-resistant mutants of *Rm. vanniellii*
using the agar tube technique

This method was adopted due to the failure of attempts using the standard plating technique. Molten PYM agar, containing antibiotics at 100 μ g/ml and held at 45°C was inoculated with exponential growth phase cells of *Rm. vanniellii* to give a suspension of 5×10^8 - 5×10^9 cells/ml. The suspension was well mixed and then poured aseptically into 10/15ml sterile test tubes which were cooled rapidly in cold water. The tubes were incubated at room temperature and illuminated from the side by a 60W tungsten bulb. In this way incubation could be continued indefinitely without changes occurring in the composition of the medium due to dehydration. Using this method, Nalidixic acid and Streptomycin-resistant mutants of both Rm5 and P+S strains were easily isolated. The colonies appeared after 5-7 days incubation. The agar in the tubes was gently blown out into a sterile petri dish by passing oxygen-free gas through a canula inserted between the tube wall and the agar. The agar columns were sectioned and the colonies restreaked on plates containing the antibiotics at the same concentration. They were found to be truly resistant and subsequently grew well, on plates containing the antibiotic, using the 'gas-bag' incubation technique.

To obtain a frequency of spontaneous mutation to various antibiotics, at concentrations likely to be used in conjugation experiments, a suspension of cells was serially diluted and aliquots of each dilution used to inoculate 10/15ml of molten medium (45°C) in 25ml universal bottles. The cells were uniformly distributed by inverting the universal a couple of times and then the medium was poured into test tubes as above. The original cell suspension was inoculated into medium

containing the selective antibiotic and the dilutions were inoculated into medium without antibiotic to calculate the viable count. Table 4.2.2 shows the frequency of spontaneous resistance to various antibiotics as determined using this method.

4.2.3 Discussion

As described above, the tube method of isolating spontaneous antibiotic mutants of Rm. vannielii worked giving mutants at a comparable frequency to that observed for other microorganisms. The enigma is why this method should work when the 'gas-bag' plate method did not. It is conceivable that the frequency of mutation is so low, less than 5.7×10^{-10} , that a large number of cells have to be applied to the surface of the plate and this in some way impedes the growth of any antibiotic-resistant mutants. There may be an initial depletion of nutrients by the general population before killing or stasis occurs. Another more likely factor is that the dehydration of the plate during incubation impedes growth.

In the agar tube the inoculum is spread throughout the volume of the medium rather than being densely spread on the surface and therefore each mutant cell is less likely to find itself in limiting conditions. In addition, the environment remains constant throughout incubation, apart from changes caused by the growth of the colonies themselves.

4.2.4 Apparatus for incubation of tubes under controlled conditions

As previously observed, the tungsten lamps must have had a heating effect. Thus, in order to provide a greater degree of control over the

temperature in the tubes of the agar, a perspex tank was constructed in which the tubes could be placed. Water at a set temperature was circulated around them using a Churchill water circulator. Using this apparatus it was possible to control temperature accurately and eliminate local variations in temperature. Light intensity was controlled by varying the number of lamps and distance from the source.

4.3 Growth of Rhodopseudomonas viridis and Rm. vannielii in tubes under phototrophic and heterotrophic conditions

4.3.1 Introduction

When first isolated Rm. vannielii was considered to be a strict photoanaerobe (Duchow and Douglas, 1949; Conti and Hirsch, 1965; Trentini and Starr, 1967a), although Pfennig (1970) demonstrated microaerophilic growth in the dark in agar deeps. Aerobic dark growth was subsequently confirmed by Dow (1974) and France (1978). Kelly (1985) suggested that the poor growth under aerobiosis and the abnormal morphology observed was due to a sensitivity to oxygen, a phenomenon found in the 'brown' species of Rhodospirillum (ie Rs. fulvum, Rs. molischianum, Rs. photometricum) (Pfennig, 1978) and Rp. viridis but not found in most other members of the Rhodospirillaceae. Kelly (1985) reported relatively good growth under limited aeration in liquid cultures, (ie microaerophilic conditions). Rm. vannielii cells grown under phototrophic conditions exhibited an endogenous O₂ uptake which suggested the existence of a respiratory system under photosynthetic growth conditions, characteristic of most members of the Rhodospirillaceae. Additional evidence for an oxidative respiratory system in Rm. vannielii was the increase in respiratory dehydrogenase

activity and the appearance of 2-oxoglutarate dehydrogenase activity under aerobic conditions, indicating the operation of a complete tricarboxylic acid cycle. Under photosynthetic anaerobic conditions however, the cycle is incomplete because of the absence of 2-oxoglutarate dehydrogenase (Morgan et al., 1986).

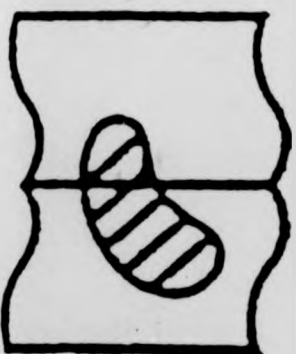
Within the Rhodospirillaceae, Rd. viridis is the most closely related to Rhodomicrobium (Gibson et al., 1979). It was found that Rd. viridis showed a sensitivity to high oxygen tension, and cultures incubated in the dark proceeded to exponential phase only if a large inoculum was used which could lower the oxygen tension sufficiently to cause microaerophilic conditions (Weyer, personal communication).

Using a solid agar tube technique, Weyer demonstrated that there was a specific depth in the agar column at which growth occurred in the dark. In this case, the surface of the agar was not overlaid with paraffin or petroleum jelly and a band of growth occurred a few mm below the surface. Above this level, growth was inhibited by high oxygen tension and below it no growth occurred because there was no oxygen or light. Again, this is similar to the 'brown' species of Rhodospirillum which show a sensitivity to oxygen that only allows microaerophilic growth under chemoheterotrophic conditions.

4.3.2 Results

In the present study, tubes of PYM agar inoculated with Rm. vannielii cells were incubated in the dark or in the light and were compared with tubes inoculated with Rd. viridis and incubated under identical conditions. The results are shown in figure 4.3.2. Rd. viridis and Rm.

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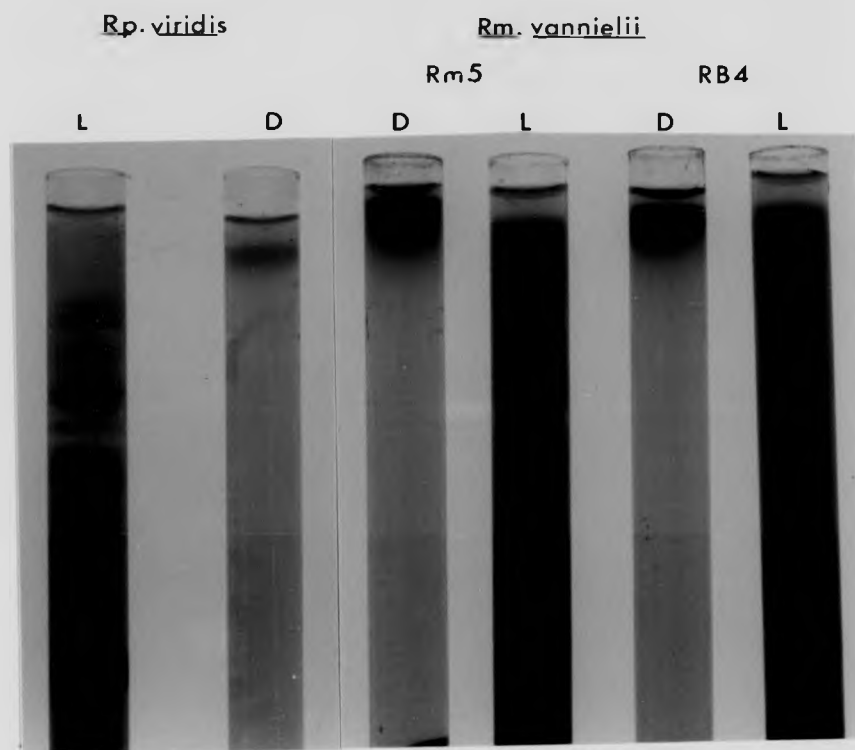


Figure 4.3.2 Comparison of growth modes of Rm.vannielii and Rp.viridis

Strains were grown in PYM agar columns under illuminated (L) and dark (D) conditions. Tubes of molten PYM agar at 45°C were inoculated from exponential phase cultures, mixed by inversion and cooled in a water bath. For dark conditions the tubes were wrapped in foil and all tubes were incubated in a 30°C perspex waterbath with illumination from tungsten bulbs.

vannielii were observed to behave very similarly: in the light both showed growth throughout the tube with a narrow band at the top of the agar column when growth was inhibited by oxygen which diffused from the surface (the tubes were not overlaid with paraffin); in the dark, both species exhibited a narrow band of growth about 2mm below the surface of the agar column, with no growth above or below it, suggesting that there was a specific region in the column in which the oxygen tension was suitable for growth to occur in the dark.

4.4 Microaerophilic growth of *Rm. vannielii* on plates

4.4.1 Introduction

Until the present study, although the ability of *Rm. vannielii* to grow microaerophilically in the dark had been recognised, the organism had not been grown on plates under such conditions. Subsequent to his demonstration of microaerophilic growth of *Rp. viridis* in tubes, Weyer had a controlled-temperature/oxygen tension/light intensity cabinet constructed in which he grew *Rp. viridis* successfully on plates under a 1% oxygen atmosphere in the dark.

4.4.2 Results

In the present study *Rm. vannielii* was found to grow well on plates incubated in the dark under microaerophilic conditions (1-4% O_2), using 'Tupperware' boxes as growth chambers as described in Section 2.7.2.2. Colonies of a desirable size were obtained after about 14 days incubation at 30°C and colony size was observed to be more uniform (both on the same plate and between plates) than had been previously found

using the 'gas-bag' incubation technique for growth in the light.

Several problems were encountered when using this technique. Occasionally a box would leak due to improper sealing causing an increase in oxygen tension which inhibited growth of colonies; the oxygen tension in boxes was regularly checked as described in Section 2.7.2.1 and regassing carried out if necessary; gross fungal/actinomycete contamination of plates sometimes occurred and this was due to growth on the inner surface of the 'Tupperware' boxes, when condensation had accumulated in the previous experiment. Presumably the plates became contaminated during gassing of the box when there would be a vigorous circulation of gas in the box; contamination was avoided by cleaning the boxes after each incubation with a small volume of industrial methylated spirit and by gassing the boxes with nitrogen at a much lower pressure. Although this method involved incubation periods of up to 14 days, it was found that the plates did not dehydrate to the extent that was observed using the 'gas-bag' incubation technique and there was no problem with condensed water accumulating on the petri dish lids.

4.4.3 Discussion

Incubation under microaerophilic conditions, using 'Tupperware' boxes, had several advantages over the incubation under anaerobic conditions in the light, using the 'gas-bag' technique, despite the longer time taken for colonies to appear. The growth conditions appear to be more constant as indicated by colony size on each plate and between plates. Using the 'gas-bag' it was difficult to ensure uniform lighting especially when working with a large number of plates. Moreover, the plates did not

dehydrate, a process which must cause a change in the growth conditions. Additionally, the temperature in the 'Tupperware' boxes is uniform whereas the heating effect of tungsten lamps causes localized heating of the 'gas-bag' making it difficult to predict what the actual temperature at the agar surface would be.

4.5 Effect of yeast extract concentration on plate growth of *Rm. vannielii* and growth on nutrient agar

4.5.1 Introduction

Purple non-sulphur bacteria are able to photoassimilate a wide variety of organic compounds, (Pfennig, 1978), and complex organic nitrogen sources are widely used to give increased growth rates. Most species have a requirement for one or several vitamins and this is in good agreement with the photoorganotrophic nature of these organisms. Such compounds are always present in habitats dominated by the active breakdown of organic matter. Since they are characteristically unable to break down organic macromolecules, they depend on the preceding activity of chemoorganotrophic bacteria which can degrade such molecules. These two types of organisms are found together in relatively high numbers in habitats where the decay of plant residues results in anaerobic conditions, and low molecular-weight breakdown products are liberated to produce growth conditions for Rhodospirillaceae. Such a situation is likely to occur in the mud of eutrophic ponds and ditches, the littoral zone of lakes, rivers and the sea, and all kinds of sewage lagoons (Pfennig, 1978). *Rm. vannielii* has been isolated from such nutrient-rich environments, although it is characteristically associated with oligotrophic environments.

Duchow and Douglas (1949), who first isolated *Rm. vanniellii* found that the organism required no added growth factors. Although a simple defined basal salts medium sustained growth, yeast autolysate was found to stimulate growth. In order to have a defined medium, which is necessary for many types of studies, Trentini and Starr (1967 a,b) narrowed the growth stimulating activity in yeast extract down to a single vitamin - riboflavin which at $1\mu\text{g ml}^{-1}$ gave the same stimulation of growth as yeast extract at 0.1% (w/v).

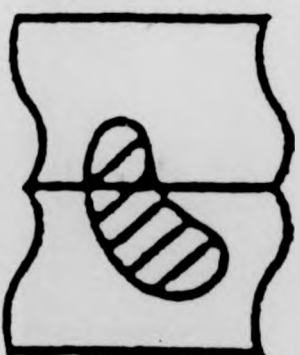
4.5.2 Results

Figure 4.5.2 shows that when yeast extract, (YE) was increased from 0.1% to 0.3% (w/v), microaerophilic growth was stimulated. Colonies of a desirable size were obtained after a 10 day incubation at 30°C rather than the 14 days previously required.

Table 4.5.2a shows the results of an experiment to define the growth-limiting concentration of YE in PYM agar. PM medium was prepared as described in Section 2.2.1 and sterile yeast extract was added from a concentrated stock solution to give a range of final concentrations between 0.1 and 1.4% (w/v).

Two strains (Rm5 and RB4) were tested for growth, under dark microaerophilic conditions, on agar plates containing this range of YE concentration. RB4 (a P+S strain) was inhibited at concentrations above 0.8%, whereas the chain-forming strain Rm5 was much less sensitive to high concentrations of YE and sustained growth at concentrations of up to 1.2%.

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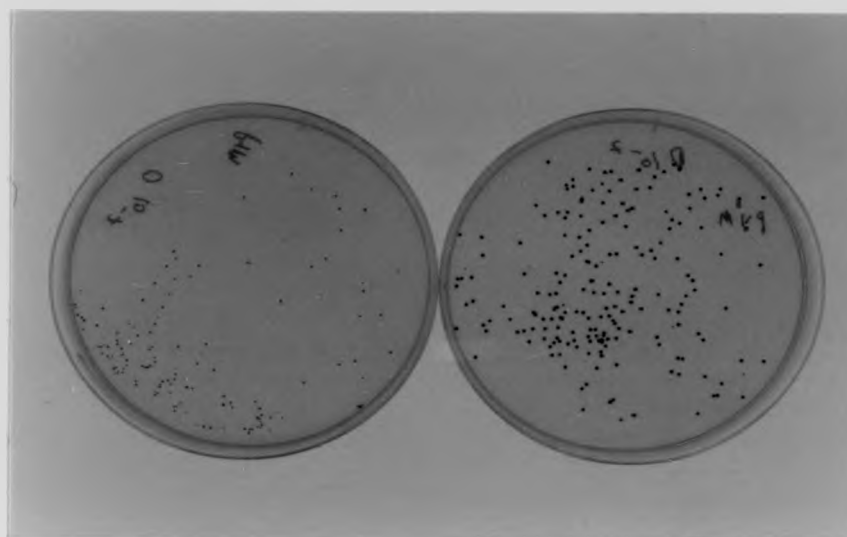


Figure 4.5.2 Stimulation of growth under microaerophilic conditions by increasing YE concentration.

Aliquots of a diluted exponential phase culture of *Rm. vannielii* RB4 were spread on plates containing 0.1 and 0.3% YE in PM medium. Plates were incubated under microaerophilic 1% O₂/dark conditions (section 2.7.2.2) and the observation above was made after 10 days incubation at 30°C.

Table 4.5.2a Determination of the growth-inhibiting concentration of yeast extract

% YE (w/v) in PM medium	Growth (+/-)	
	Rm5	RB4
0.1	+	+
0.2	+	+
0.4	+	+
0.8	+	+(reduced)
1.0	+	-
1.2	+	-
1.4	-	-

Exponential phase cultures of Rm.vannielii Rm5 (chain forming) and RB4 (P + S, Nal^r) were streaked out on plates of PM agar containing varying concentrations of YE. Plates were incubated under microaerophilic/dark conditions (section 2.7.2.2). (+ = growth; - = no growth).

Table 4.5.2b Determination of the growth-inhibiting concentrations of nutrient broth in nutrient agar

Conc. of NB $g\ l^{-1}$	% of normal NA	Rm5		RB4	
		growth	colony size (mm)	growth	colony size (mm)
13	100	+(in well only)	0.1	-	-
11.7	90	+	0.5	+(in well only)	0.1
10.4	80	++	0.5 - 1.0	++	0.5
9.1	70	+++	1.0	++	0.5
7.8	60	+++	1.25	+++	1.0
6.5	50	++	0.5 - 1.0	+++	1.0
4.3	33.3	++	0.5	++	1.0
3.25	25	+	< 0.5	+	0.5

Exponential phase cultures of Rm.vannielii Rm5 (chain forming) and RB4 (P + S, Nal^r) were streaked out on agar plates containing varying concentrations of nutrient broth. Plates were inoculated under microaerophilic/dark conditions (section 2.7.2.2) and inspected after 10 days. (+ = weak growth; +++ = strong growth; - = no growth). Colony size was estimated visually by comparison with a standard ruler with mm divisions.

As a result of these observations, it was decided to test Rm. vanniellii growth on NA since this is simpler to prepare than PYM, and for the purpose of genetics a defined medium is not essential. Table 4.5.2b shows the effect of NB concentration on the growth of the two strains Rm5 and RB4 when grown on NA plates in dark microaerophilic conditions. Rm5 grew well on NA made with 60/70% the recommended concentration of 'Oxoid' NB used for making NA (normally 13gL^{-1} NB). Colonies were observed to decrease in size as the concentration of NB increased to 100% or decreased to 25%. Thus the high concentrations would appear to inhibit growth and the lower concentrations limit growth presumably by nutrient shortage. A similar pattern was observed when growing RB4 on NA containing the same range of concentrations of NB. Again the maximum colony size was observed at 60% of the normal concentration NB. However, this strain seemed to be more sensitive than Rm5 to the high concentration of NB, being inhibited completely at 100% (13gL^{-1}); and less affected by nutrient shortage at the lower concentration of NB.

4.5.3 Discussion

The reduction of the incubation time required to obtain workable colonies from 14 days to 10 days by increasing the YE concentration is a big advantage. Apart from reducing experiment time, it also means that there is less time for plates to dehydrate or antibiotics to decompose. Subsequent to this discovery, medium containing 0.3% (w/v) YE was routinely used.

As shown here it would also be possible to use a 60% normal concentration NA for the growth of Rm. vanniellii. This would be an

additional time saver since the medium is easily prepared, does not involve alterations of pH, and mistakes are less likely to be made in its preparation. There is also the possibility that the different sensitivity to concentration of NB in the medium displayed by the chain forming and 'P+S' strain could be exploited as a selection system for developmental mutants.

Given that this organism can be isolated from nutrient-rich habitats, (assuming that it grows actively in such habitats), it is perhaps not surprising that it can grow on such complex media.

4.6 Definition of the minimum-inhibitory-concentration (MIC) of various antibiotics during plate growth of Rm. vanniellii

4.6.1 Introduction

When carrying out simple genetical experiments, eg conjugation to transfer a broad host-range plasmid, with a genetically 'virgin' organism, it is desirable to know the MIC of various antibiotics used in selection of the desired transconjugants. It cannot be assumed that the level of resistance provided by the presence of the antibiotic resistance gene (on a plasmid or transposon) in the recipient would be the same as that conferred to the donor strain. This could be due to a number of factors including differences in plasmid copy number, different efficiency in recognition of gene promoters or different activity of the gene product in the two organisms.

4.6.2 Results

The MIC of various commonly used antibiotics was defined using the microaerophilic dark growth conditions. Two strains RB1 and RB4, (Nalidixic acid-resistant, chain forming and P+S strains respectively), were tested for growth on a number of antibiotics over a range of concentrations between 1 and $25\mu\text{g ml}^{-1}$ as shown on Table 4.6.2 Both strains were found to be sensitive to all six antibiotics used (Ap, Sm, Km, Cm, Tc and Sp) at concentrations as low as $5\mu\text{g ml}^{-1}$. However, both strains exhibited growth in the inoculation well when streaked on plates containing Cm at $5\mu\text{g ml}^{-1}$. Both strains were insensitive to Ap, Cm and Sm at $1\mu\text{g ml}^{-1}$ with single colonies growing all over the plate but appeared to be sensitive to Sp, Km and Tc at this concentration as growth was restricted to inoculation wells. The M.I.Cs would therefore appear to be between 1 and $5\mu\text{g ml}^{-1}$ for Ap, Sp, Km, Tc and Sm and between 5 and $10\mu\text{g ml}^{-1}$ for Cm.

4.7 Conclusion

The 'gas-bag' incubation technique was found here to be unsuitable for the types of manipulations that are required for genetic experiments with *R. vannieli*. It is suggested that this was due to the inability to predict the temperature at the agar surface, provide uniform illumination and prevent dehydration of plates.

For some manipulations (eg selection of antibiotic-resistant mutants or other low frequency events), which do not involve the picking of large numbers of colonies, the agar tube method provides a good alternative allowing incubation in the light under controlled conditions.

Table 4.6.2 Determination of the minimum-inhibitory-concentrations of antibiotics during plate growth

Antibiotic	Concentration $\mu\text{g ml}^{-1}$	Growth	
		RB1	RB4
Ap	1	+	+
Ap	5	-	-
Ap	10	-	-
Ap	15	-	-
Ap	25	-	-
Sp	1	+/-	+/-
Sp	5	-	-
Sp	10	-	-
Sp	15	-	-
Sp	25	-	-
Km	1	+ in well	+ in well
Km	5	-	-
Km	10	-	-
Km	15	-	-
Km	25	-	-
Cm	1	+	+
Cm	5	+ in well	+ in well
Cm	10	-	-
Cm	15	-	-
Cm	25	-	-
Tc	1	+ in well	+ in well
Tc	5	-	-
Tc	10	-	-
Tc	15	-	-
Tc	25	-	-
Sm	1	+	+
Sm	5	-	-
Sm	10	-	-
Sm	15	-	-
Sm	25	-	-

Exponential phase cultures of *Rm. vanielii* RB1 (Rm5 , Nal^{r}) and RB4 ($\text{P} + \text{S}$, Nal^{r}) were streaked out on PM agar plates containing 0.3% YE and antibiotics at a range of concentrations. Plates were incubated under microaerophilic/dark conditions (section 2.7.2.2). (+ = growth; - = no growth).

For plate growth, microaerophilic/dark incubation was found to yield better results. Despite a longer incubation period it gave reproducible growth conditions. Yeast extract (0.3% (w/v)) stimulated growth under microaerophilic/dark conditions considerably (compared to 0.1% (w/v) YE), reducing the time required to obtain workable colonies.

NA, containing a reduced concentration of NB, is a possible alternative growth medium for carrying out genetic manipulations of Rm. vanielii.

CHAPTER 5

NITROSOGUANIDINE AND ULTRAVIOLET LIGHT MUTAGENESIS

5.1 Introduction

The aim of this work was the development of a system for genetic analysis in this genetically uncharacterized organism. If successful, this system would later be applied to the analysis of the two major, inter-related processes under investigation in Rm. vannielii:- cell differentiation in the swarmer cell cycle and development of the photosynthetic apparatus.

The first step in any genetic analysis is the isolation of mutants that are defective in the process of interest; eg Bacillus sporulation mutants unable to form spores or conditional-lethal mutants of E. coli unable to complete the cell cycle under non-permissive conditions. The ability to obtain mutants and study gene transfer make it potentially possible to investigate any given process to the level of gene expression.

The range of mutant types that can be isolated from the Rhodospirillaceae was adequately reviewed by Saunders (1978) and this included the following classes: antibiotic/heavy metal-resistant mutants, pigment mutants, electron transport mutants, temperature-sensitive mutants and morphological mutants. Thus a wide range of mutants have been isolated, most commonly from Rb. sphaeroides, Rb. capsulatus and Rs. rubrum. Typically, mutants were obtained by ultraviolet (UV) light or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis essentially by the method of Adelberg et al (1965). Marrs et al (1980) described methods for the isolation of mutants of rhodospseudomonads and asserted that these organisms present no special

difficulties with respect to mutagenesis or selection techniques that are routinely applied to other microorganisms.

Marrs (1978) described the ways in which mutants may be advantageously used in solving bioenergetic problems inherent in the study of bacterial photosynthesis.

Research into bacterial differentiation and membrane development using Rm. vannielii has progressed as described in Section 1.4. However, in recent years progress has been limited by the unavailability of any mutants specific for these systems, indeed by the lack of any mutants at all, except for some antibiotic-resistant isolates (Potts, 1980; Scott and Dow, 1987). A wealth of biochemical and physiological techniques have been applied or developed in Rm. vannielii and these techniques could be immediately applied to the investigation of mutants, thus yielding a great deal of information about biochemical processes operating in the wild type.

Since UV light-mutagenesis and NTG-mutagenesis generally work well in other members of the Rhodospirillaceae, these mutagens were the ones of choice in this study. This chapter summarizes attempts to carry out mutagenesis using both of these techniques and describes the types of mutant that were isolated as a result.

5.2 UV light-mutagenesis

5.2.1 Introduction

DNA absorbs UV light strongly, with an absorption maximum of about

260nm. Cells are rapidly killed and a high rate of mutation is observed amongst the survivors. The UV dose at which maximal mutations are induced must be determined experimentally for each strain or organism, as it can vary from a dose corresponding to a survival level of 50% down to less than 0.1%. Much, if not all, of the mutagenic activity of UV light is attributable to the formation of pyrimidine dimers (ie covalent bonds formed between pyrimidine residues adjacent to each other on the same strand of DNA).

5.2.2 Results: UV-irradiation followed by 'gas-bag' incubation

UV-mutagenesis of RB4 was performed as described in Section 2.1.4. The irradiated suspensions were diluted and plated out, without expression time, on PYM plates containing either Nalidixic acid at $100\mu\text{g ml}^{-1}$ or Nalidixic acid plus Streptomycin both at $100\mu\text{g ml}^{-1}$, to select for any antibiotic-resistant mutants that might be induced. Plates were incubated using the 'gas-bag' technique using tungsten lamps on the laboratory bench to avoid over-heating. Colonies were counted and inspected after 6 days incubation. Figure 5.2.2 shows the killing curve obtained. The curve appears to be bi-phasic with an initial period, 0-60 sec (43% of the population killed), in which the rate of killing is slower than that observed in the second period, 60-100 sec (95% of the initial survivors killed). The rate of killing in the second period was more than double that in the first. This may indicate the operation of some type of repair mechanism, eg photoreactivation. At the end of irradiation, (100 sec), the level of survival was 1.67%. Some variation in size of colonies on the plates was observed but no pigment or colony-morphology mutants were found. Great variation in colony size was observed between plates, including the controls and this was probably

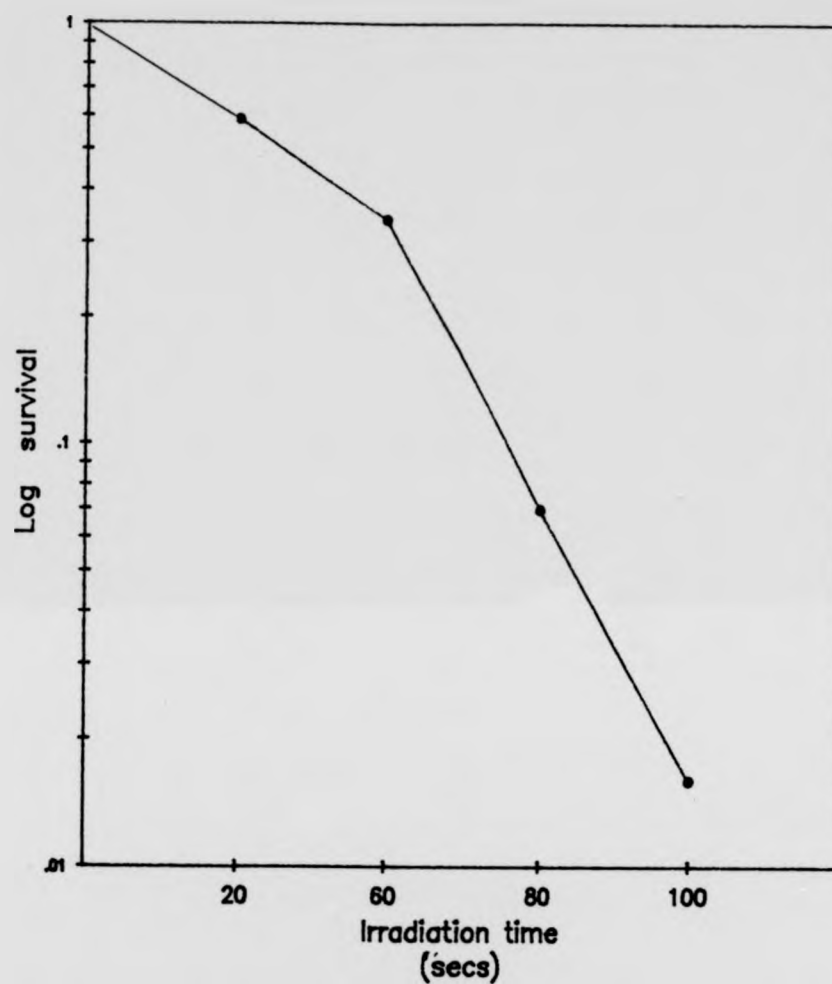


Figure 5.2.2 UV-mutagenesis of *Rm. vanniellii* RB4; 'gas-bag' incubation

Mid-exponential growth phase cells were resuspended in PBS buffer and samples exposed to UV-light for a range of time periods as described in Section 2.1.4. Plating on PYM (YE 0.3%) was carried out immediately after irradiation and dilution. Colonies of survivors were counted after 6-days incubation in 'gas-bags' under tungsten lamps.

due to the incubation technique as discussed in Chapter 4. This type of experiment involves the use of many plates and it is very difficult to provide uniform illumination and temperature control. In addition, no antibiotic-resistant mutants were observed on plates containing Streptomycin nor were there any spontaneous resistant mutants on control plates.

5.2.3 Results: UV-irradiation followed by microaerophilic incubation

Figure 5.2.3a(1) shows the results of the same mutagenic treatment followed by microaerophilic incubation of plates. As before, no expression time was used and no attempt was made to isolate antibiotic-resistant mutants. As before, the killing curve was bi-phasic and 1% of the survival occurred at 60-80 sec irradiation. In a control, in which cells were subjected to the same procedure without irradiation, no loss of viability was detected, (Figure 5.2.3a(2)). There was a lot of variation both in size and colour of colonies grown from irradiated suspensions and a few rough colonies resembling that of the chain-forming Rm5 strain were observed. Colonies of RB4 (a P+S derivative) are round, smooth and shiny whereas colonies from a chain forming strain are irregular in shape, dry and rough in appearance (Figure 5.2.3c) (Dow and France, 1980). Optical microscopy revealed that there were chains of cells present in the 'revertant' colonies which grew after irradiation.

Under these incubation conditions the colony size is fairly uniform and Figure 5.2.3b(2) shows that the number of smaller-than-average colonies was constant at about 5% in the non-irradiated control; for irradiated populations the number of small colonies increases steadily from 5% to a maximum of 50% at 80 sec, after which it decreases steadily as the

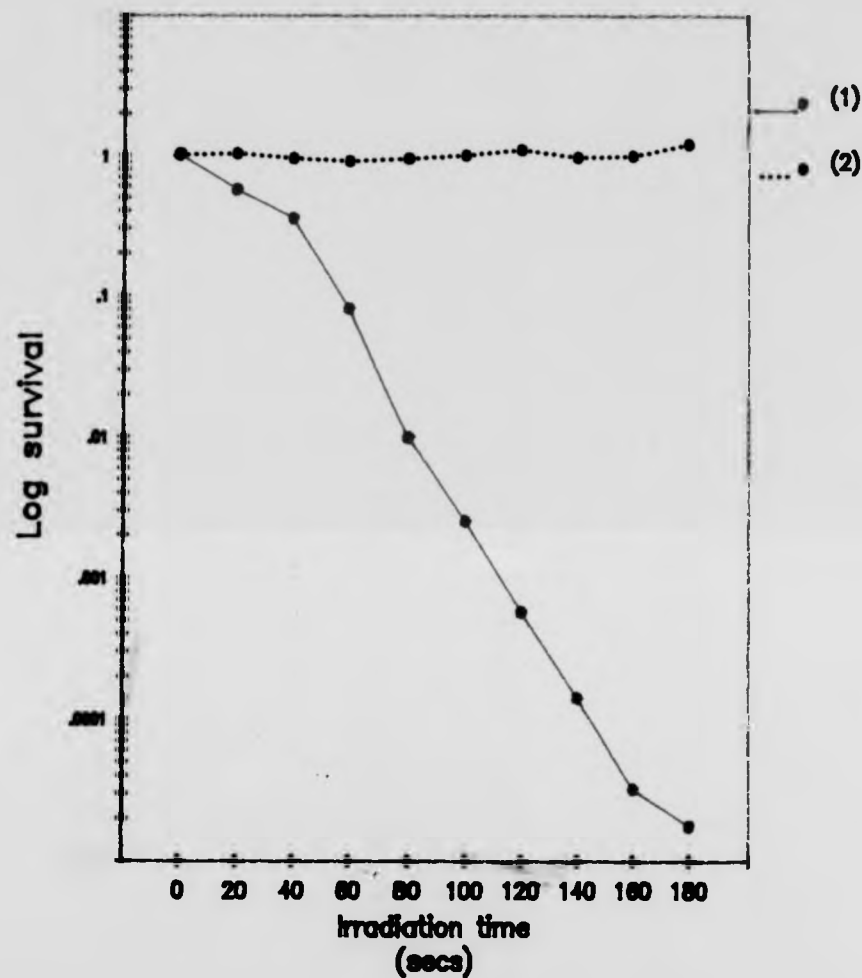


Figure 5.2.3a UV-mutagenesis of *Rm. vannielii* RB4; microaerophilic incubation

Irradiation of exponentially growing cells resuspended in PBS buffer was performed as described above. Cells were plated on PYM (0.3% YE) and incubated under microaerophilic 1% O_2 , dark conditions for 10-14 days. The killing curve obtained (1) is compared to a control in which cells were subjected to the same process without irradiation (2).

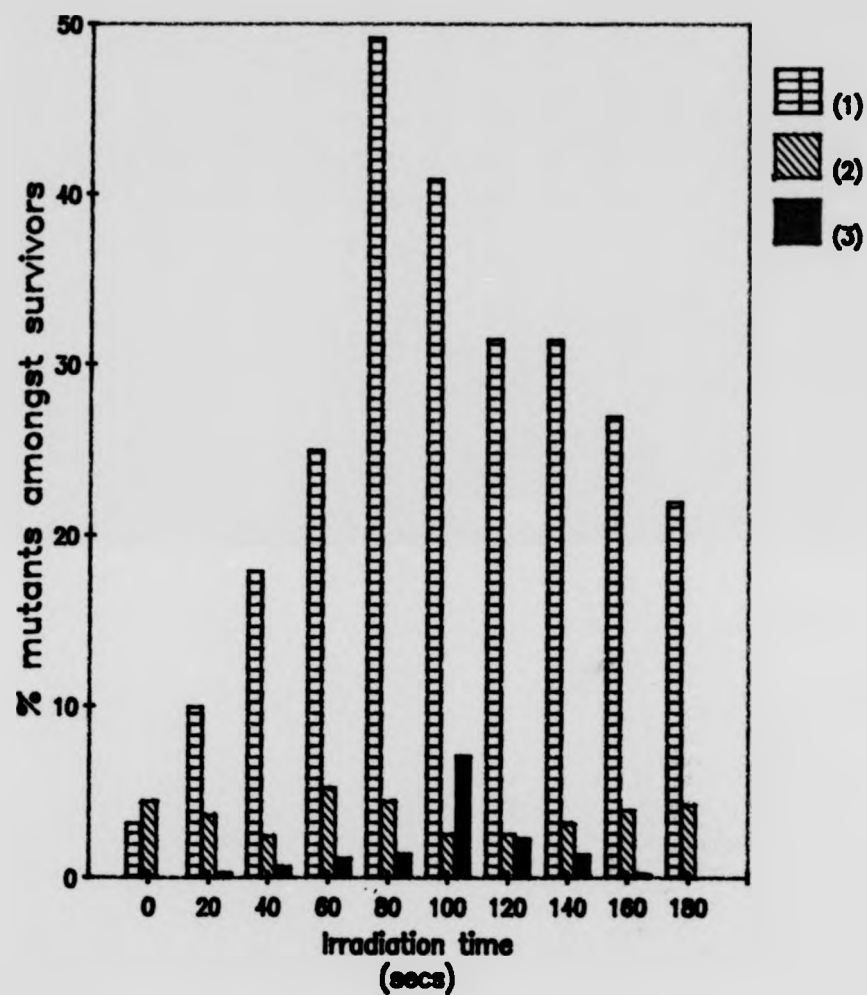


Figure 5.2.3b Variation in colony size and pigmentation after
UV-mutagenesis

- (1) 'smaller-than-average' colonies on plates spread with irradiated cells
- (2) 'smaller-than-average' colonies on control plates - (non irradiated cells)
- (3) colonies with altered pigmentation on plate spread with irradiated cells

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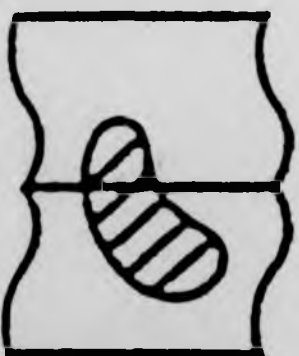




Figure 5.2.3c Smooth, round, shiny colonies of a P + S strain compared to the irregular, rough, matt colonies of a chain forming strain.

(from France 1978)

population is killed (Figure 5.2.3b(1)).

Figure 5.2.3b(3) shows the number of colonies with altered pigmentation on the same plates as above. The pattern was the same as that observed for numbers of small colonies although the total number of pigment mutants was much smaller. The number of pigment mutants increased from none at times 0 and 20 seconds to about 4% of the survivors at 100 sec, thereafter the number decreased steadily to zero at 160 and 180 sec.

5.2.4 Discussion

It is difficult to understand why no mutants were observed using the 'gas-bag' technique when the alternative mode of growth yielded so many. Although some of the pigment mutants are likely to be photosynthetically-incompetent and therefore would only grow under microaerophilic conditions, this is unlikely to be the case for all of them. Other types of mutants were produced in the first attempt, as suggested by the variation in colony size, but, once again, interpretation of this was confused by general variation caused by incubation conditions. However, both methods produced a similar bi-phasic killing curve. As suggested above, this may indicate the operation of some type of repair mechanism, eg photoreactivation which is common in other organisms. Exposure to long wavelength UV light or the shorter wavelengths of the visible spectrum, (sunlight, fluorescent-tube lighting), during the period immediately following irradiation can enzymatically reverse a proportion of the mutagenic and killing effects of a given UV dose, (Hopwood, 1970). Alternatively, there may be a difference in sensitivity of single swarmer cells and paired or stalked cells to UV-irradiation. It is known that the nucleoid structure in

swarmers is much more condensed than in stalked cells, (Dow *et al.* 1983), and this could conceivably afford some protection. Of most importance is the fact that the method yielded mutants and should therefore be valuable in obtaining mutants for specific research purposes in future. Potential photoreactivation effects, and the effects of expression time on mutant recovery were not investigated.

5.3 NTG-mutagenesis

5.3.1 Introduction

NTG is one of the most potent mutagens known, producing, under the right conditions, extremely high mutant yields with comparatively little killing. Using *E. coli*, up to 50% of the survivors of NTG treatment may be auxotrophic (Adelberg *et al.* 1965). The most common result of NTG treatment appears to be G:C to A:T transitions, although some transversions have also been observed (Miller, 1972).

The action of NTG appears to be centred specifically at the DNA replication fork. It has been demonstrated that NTG-mutagenesis can be directed at specific regions of the chromosome by synchronizing the DNA replication cycle and exposing the cells to NTG at the moment the replication fork is passing the target region. Unfortunately selective mutagenesis at the replication fork implies that any multiple mutations induced by NTG will be closely linked, and this is undesirable in most cases. Guerola *et al.* (1971) calculated that the replication region in *E. coli* is about 220-fold more sensitive to NTG mutagenesis than a similar length of the rest of the chromosome.

5.3.2 Results: NTG-mutagenesis of swarmer cells during the cell cycle

Initially, mutagenized cells were plated out using the 'gas-bag' technique and later the microaerophilic incubation technique was used. In both cases the mutagenesis procedure was essentially that described in Section 2.1.3. Figure 5.3.2a compares the killing curve of swarmer cells at the beginning of the swarmer cell cycle, with the killing curve of cells, obtained from the same culture as the swarmers, 2½ hrs into their developmental cycle. An homogeneous population of swarmers was prepared as described in Section 2.11 and as the population passed through its differentiation sequence it was treated with NTG, at 100 µg ml⁻¹, at times 0 (swarmer cells) and 2½ hrs (stalked cells). Differentiation was monitored using coulter counter/cell volume distribution analysis and optical microscopy. The cell volume distribution analysis at 0 hr and 2½ hr is shown in Figure 5.3.2b. The traces indicate that the population differentiates fairly synchronously as the peak shifts towards greater cell volume. The base of the peak becomes broader because a certain proportion of the population in any synchrony experiment fail to differentiate. It is therefore not possible to say that one killing curve is characteristic of swarmers and the other of stalked cells. The time 0 population would contain cells that are actively differentiating in the first 30 min of the cycle and some cells that have failed to differentiate; the 2½ hr population would contain cells that have gone to the stalked stage of the cycle plus a similar number of non-differentiating cells as in the time 0 population. There is a definite difference between the two killing curves during the first 30 min: for the time 0 cells it is fairly linear whereas during this same period the 2½ hr population is killed much more rapidly and

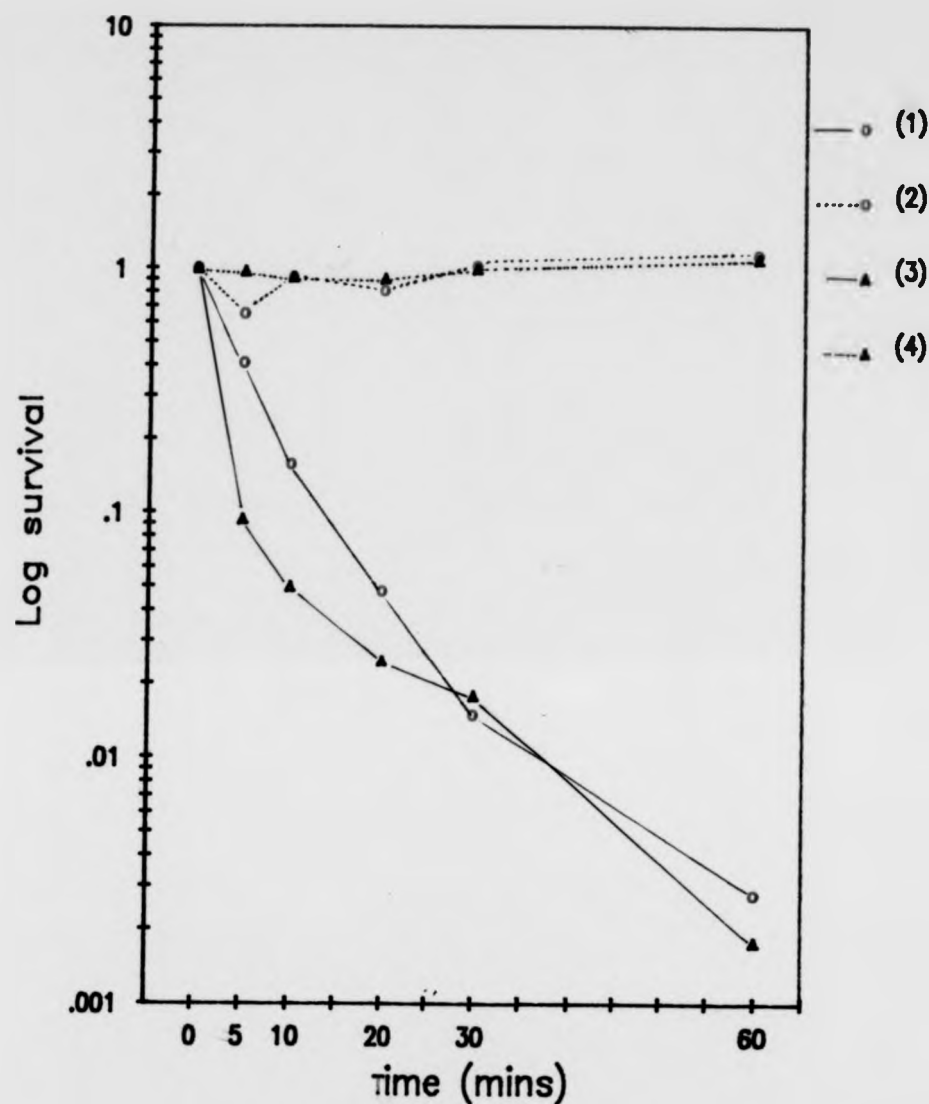
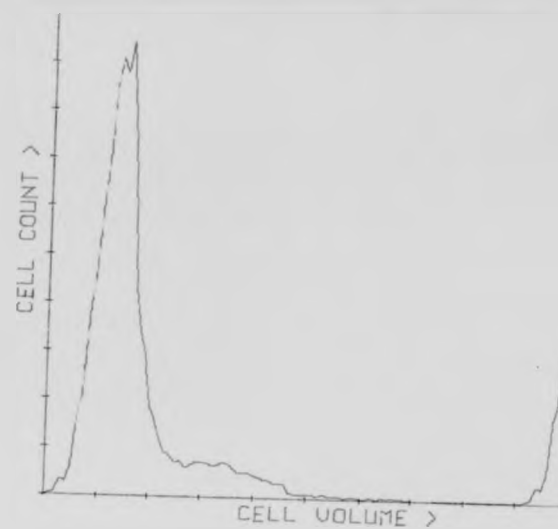
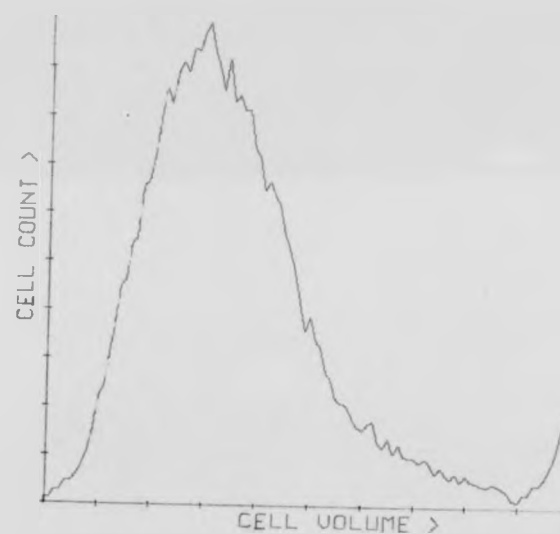


Figure 5.3.2a NTG-mutagenesis of *Rm. vannielii* Rm5 swarmer cells during the cell cycle

4 x 50 ml aliquots of swarmer cells in foil-covered, 'suba-sealed' and N_2 -flushed, 400 ml bottles were equilibrated at $30^\circ C$. To start the cell cycle the foil was removed and the bottles placed under tungsten lamps. NTG ($100 \mu g ml^{-1}$) was added at times 0 (1) and 24 hrs (3) and samples removed to determine viable counts over the following 60 min. Viable counts were also determined for non-mutagenized cells subjected to the same process (2) and (4). After washing, cells were expressed for 6 hrs (phototrophic conditions) before plating and 'gas-bag' incubation.



0hr



2.5hrs

Figure 5.3.2b Cell volume distribution analysis of the population of swarmer cells used for NTG-mutagenesis, at times 0 and 2½ hrs into the synchrony

the curve is non-linear. In the 30-60 min period both curves are similar and it is possible that both curves are bi-phasic and that this reflects the content of the population.

The controls, in which the cells were treated in exactly the same way without exposure to NTG, rules out the possibility of killing due to sensitivity to oxygen which the cells would have encountered during washing and plating out (Figure 5.3.2a (2 and 4)).

After 7 days incubation, using the 'gas-bag' technique under tungsten bulbs on the laboratory bench, the colonies grown from mutagenized cells showed considerable variation in size, pigmentation and morphology. In contrast the non-mutagenized controls, which exhibited the normal variation in size encountered using the incubation technique, had normal pigmentation and morphology. In addition to the colony variations caused by incubation conditions, chain-forming strains were found to exhibit variation in colony size due to the fact that a colony forming unit (cfu) could be a single cell or a raft/microcolony of intertwined chain cells. For these reasons, no attempt was made to assess the frequency of induction of mutants using colony size in this experiment. A representative selection of abnormal colonies was transferred to liquid cultures and grown up in anaerobic light conditions, before being reisolated as single colonies. In a screen for possible auxotrophs, 500 well-isolated colonies were picked directly from the original plates and patched onto PM and PYM (YE 0.1% w/v) plates but no auxotrophs were found.

5.3.3 Discussion

The most important point in the context of this project is that mutants were observed and isolated as a result of treatment with NTG. It is interesting that this method yielded pigment mutants when UV mutagenesis followed by 'gas-bag' incubation yielded none. This may reflect the potency of NTG as a mutagen in that it can produce extremely high mutant yields with comparatively little killing. That no auxotrophs were isolated may reflect a technical problem with their isolation rather than indicate that none were induced. One possibility is that photo-reactivation was occurring and future experiments should include 'repair time' in the dark to eliminate this possibility.

Despite the heterogeneity of the populations used, and the fact that the data are drawn from one experiment only, it is possible to make the following tentative deductions from the killing curves. The differences between the killing curves may reflect a relative impermeability of the swarmer cell envelope to that of cells that are in the differentiation sequence. A number of observations, including differential sensitivity to inhibitory agents (Scott *et al.* 1987; Whittenbury and Dow, 1977), and the differences observed between swarmer cells and budding cells when carrying out *in vivo* labelling of RNA (Potts and Dow, 1979; Dow *et al.* 1983) and DNA (Potts and Dow, 1979), have suggested that there may be a change in membrane permeability properties during differentiation. There are dramatic changes in the cell envelope during the maturation phase of the cycle when the cell loses its flagella, and there is localized disruption at the growing point during prosthecum and bud formation.

DNA replication is not thought to occur in swarmer cells, the nucleoid being in a condensed state (Whittenbury and Dow, 1977). Since NTG acts specifically at the DNA replication fork, the question arises as to how

NTG affects killing and mutagenesis in such cells. An experiment that would be interesting in this respect would be to carry out NTG treatment of swarmer cells that were held in the dark and therefore not differentiating. The permeability question could be answered by using a range of concentrations of NTG, and if DNA replication does not occur in swarmer cells then there should be little or no killing irrespective of NTG concentration. In addition, since populations of swarmer cells can be synchronized, it should be possible to use NTG mutagenesis to map the chromosome since genes with high co-mutation frequencies must be closely linked (Guerola *et al.* 1971).

5.3.4 NTG-mutagenesis of RB4 using microaerophilic selection

The 'pairs and swarmers' strain RB4 is a convenient one to use in mutagenesis experiments, since one cfu represents a single cell or a pair of cells, rather than rafts of chain cells or microcolonies formed by chain-forming strains. RB4 is Nalidixic acid-resistant, and this can be used to select against contaminants during the long incubation periods and to verify the identity of any mutants isolated.

Figure 5.3.4a(1) shows a killing curve obtained after treatment of RB4 with NTG at $100\mu\text{g ml}^{-1}$ as described in Section 2.13. In this case the cells were given expression time of 6 hr in phototrophic conditions and incubation of plates was carried out using the microaerophilic technique. The curve had the following features: there was a constant decrease in viability from time zero to 120 min and 50% killing occurred at about 5 min treatment with NTG. The control showed that there was no loss of viability during the 'handling' in air and resuspension in buffer (Figure 5.3.4a(2)). As a result of using the microaerophilic

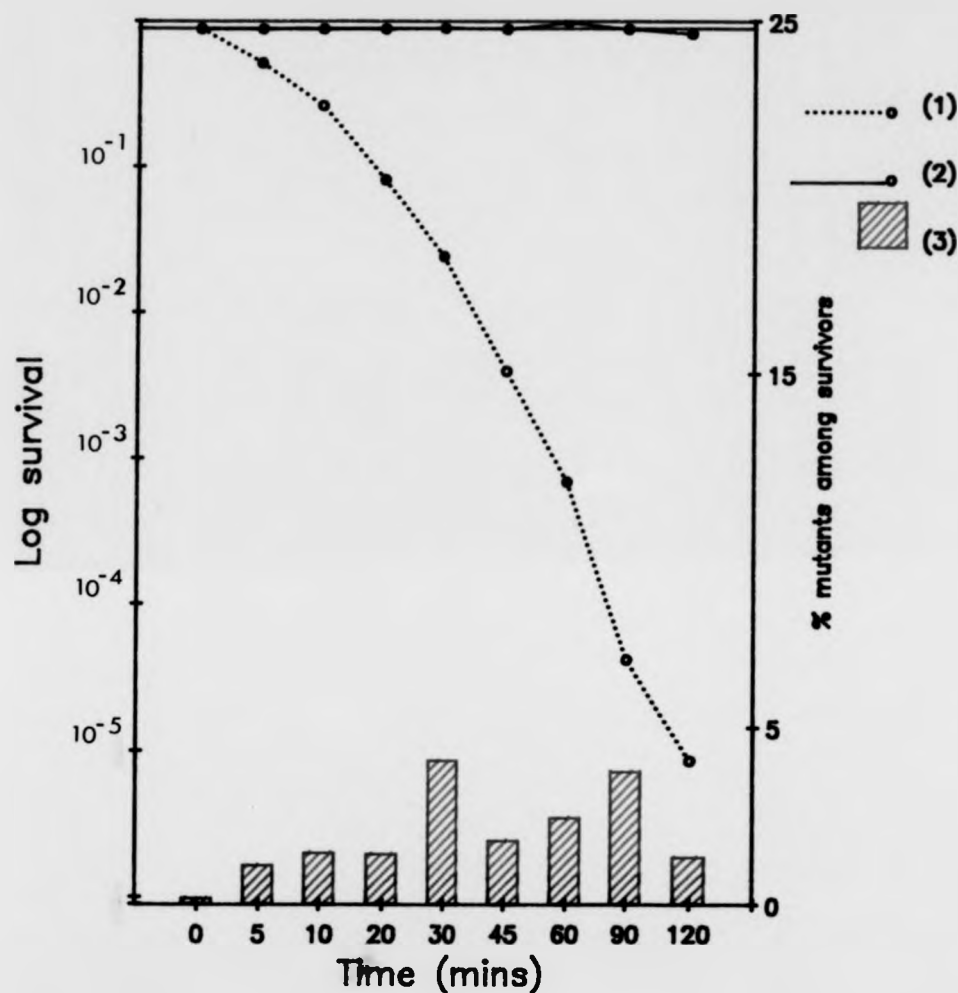


Figure 5.3.4a NTG-mutagenesis of *Rm.vannielii* RB4 using microaerophilic selection

- (1) killing curve for culture treated with NTG ($100 \mu\text{g ml}^{-1}$)
- (2) control in which cells were subjected to the same process without addition of NTG
- (3) % colonies with altered pigmentation on plates spread with NTG-treated cells. Samples were taken from an exponentially-growing culture at various times after addition of NTG, washed and expressed in fresh medium under phototrophic conditions for 6 hr. Survivors were grown up on PYM plates using microaerophilic/dark growth conditions.

incubation method many colonies with altered pigment content were observed. Typically, some pigment mutants of the Rhodospirillaceae are photosynthetically- incompetent (and some are actively killed in the presence of light and oxygen due to photooxidation). Such mutants would not be isolated unless the alternative growth mode was used. Figure 5.3.4a(3) shows the number of pigment mutant observed for each treatment. The general pattern was an initial increase from zero to a maximum of 4% of the survivors followed by a decrease as the population was totally killed. A number of colonies were selected from plates for purification and characterization as described in Chapter 6.

Apart from pigmentation, variation in colony size was observed. This variation was much greater than that on control plates. This strain forms very uniformly-sized colonies as a result of 1 cfu arising from 1 cell or a pair of cells. The colonies from this experiment were analyzed using an Artek colony counter (Model 880 Artek System Corporation, Farmingdale, NY) on which parameters can be set to count all colonies above a given size. Assuming that mutant colonies will generally be reduced in size, due to being 'unfit', counting the number of colonies in a given reduced size range (eg between 0.2mm and 0.4mm diameter), the percentage of the population represented by this size may be calculated. This may be used as an assay for the effectiveness of mutagenesis.

$$\frac{(\text{No of colonies} > 0.2\text{mm}) - (\text{No of colonies} > 0.4\text{mm})}{(\text{No of colonies} > 0.2\text{mm})} -$$

$$\frac{(\text{total no of colonies}) - (\text{No of normal size})}{(\text{total no of colonies})} -$$

$$\frac{(\text{colonies of reduced size})}{(\text{total no of colonies})} - \frac{\text{mutants}}{\text{whole population}}$$

Figure 5.3.4b(1) shows the analysis of the plates from the experiment on

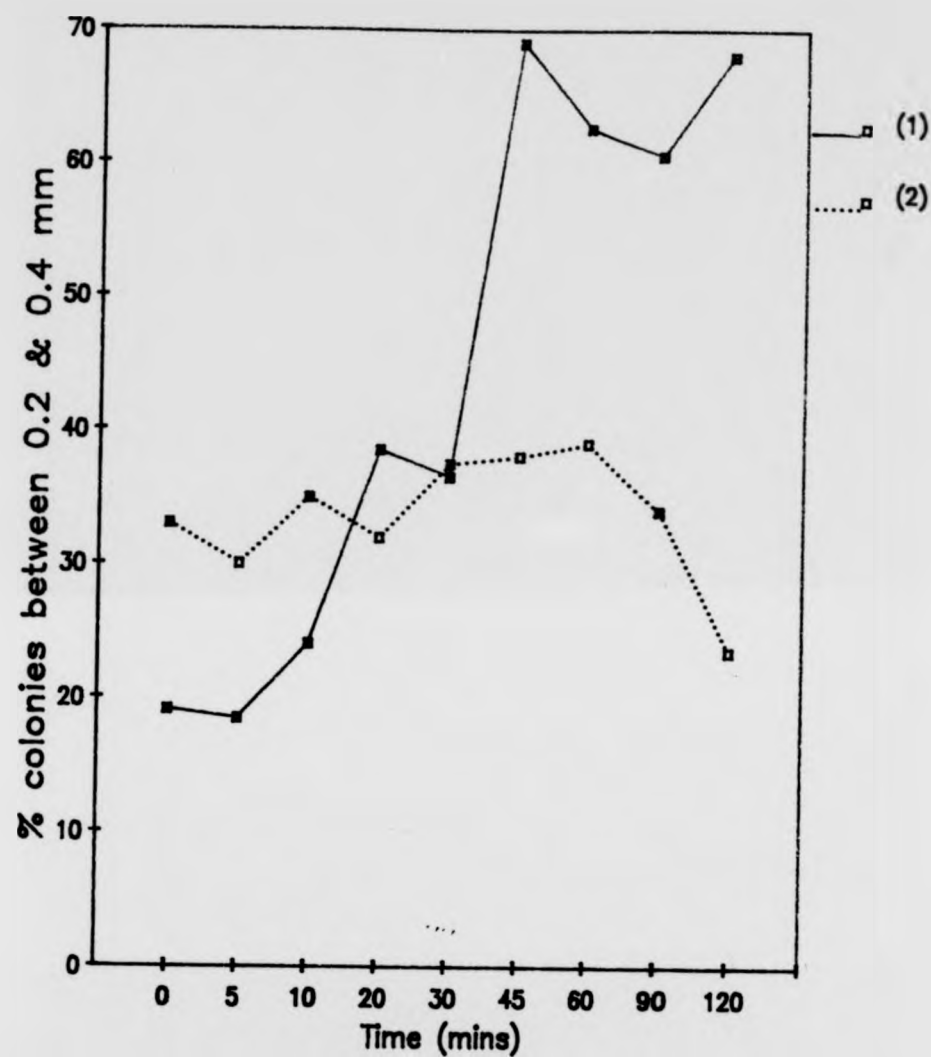


Figure 5.3.4b Analysis of colony size on plates from the NTG-mutagenesis experiment using the Artek colony counter to count colonies in the 0.2 - 0.4 mm range

- (1) colonies on plates spread with NTG-treated cells
- (2) colonies on plates spread with non-mutagenized cells

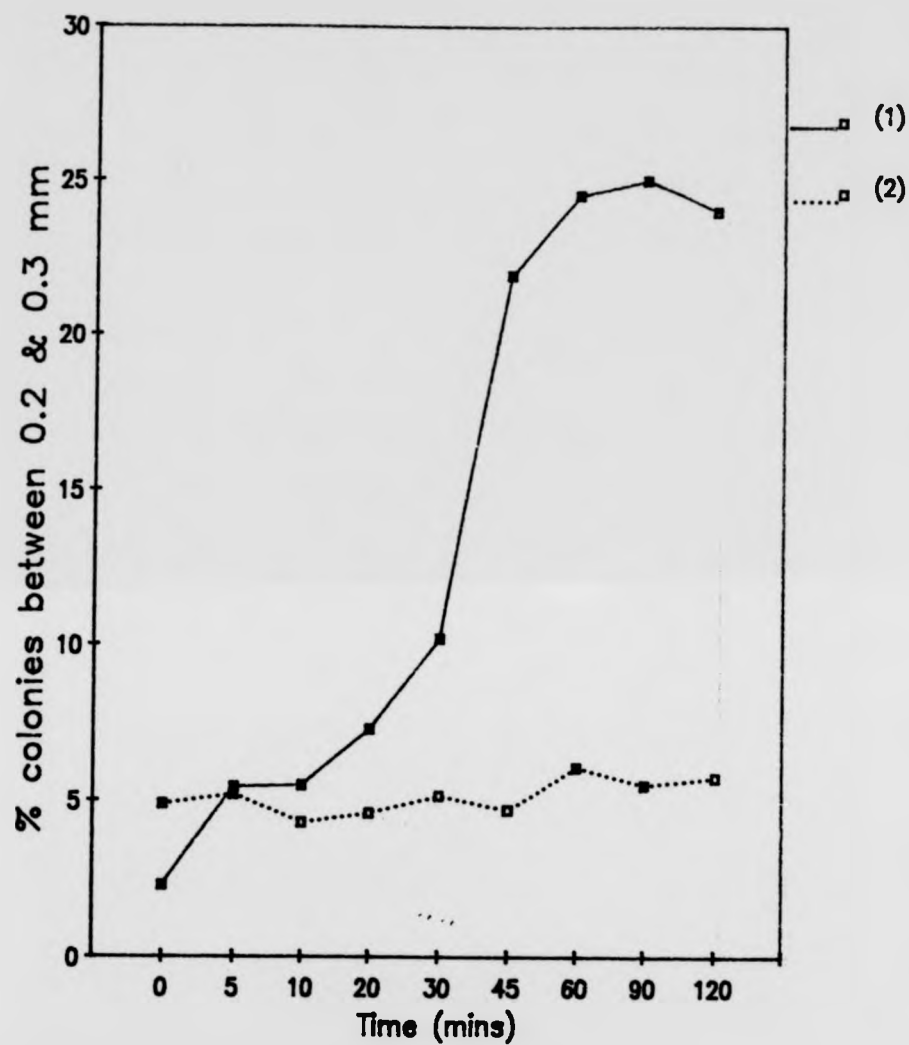


Figure 5.3.4c Analysis of colony size on plates from the NTG-mutagenesis experiment using the Artek colony to count colonies in the 0.2 - 0.3 mm range

- (1) colonies on plates spread with NTG-treated cells
- (2) colonies on plates spread with non-mutagenized cells

the previous page when the colonies between 0.2mm and 0.4mm were counted. Increased time of exposure to NTG produced increased numbers of colonies in this reduced size range, with a maximum of about 70% being observed at 45 min. Thereafter the number remained above 55% of the population for times up to 120 min. This was significantly different from the control plates where the number of colonies in this size range was never found to be above 40% and generally about 30-40% of the population (Figure 5.3.4b(2)). Figure 5.3.4c(1) shows the same analysis when the colonies between 0.2mm and 0.3mm are counted, ie only very small colonies. The curve demonstrates very clearly that NTG treatment was causing an increase in the frequency of small colonies. From time 0 the number increases from 5% to 25% at 45-60 min. After 60 min the number of colonies of this reduced size remained constant at about 24%. Analysis of the control plates showed that the number of colonies of this small size remained constant at about 5% of the total population (Figure 5.3.4c(2)).

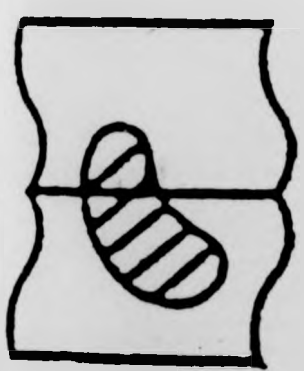
5.4 Types of mutants isolated

As a result of the experiments outlined here and in subsequent experiments using similar methods of mutagenesis, with various screening techniques, a range of different mutant types were isolated.

5.4.1 Pigment mutants

A large number of pigment mutants were isolated after both NTG and UV mutagenesis. A selection of mutants obtained after UV mutagenesis is shown in Figure 5.4.1. This was easily achieved since most of them were easily identified on plates and the frequency of isolation of certain

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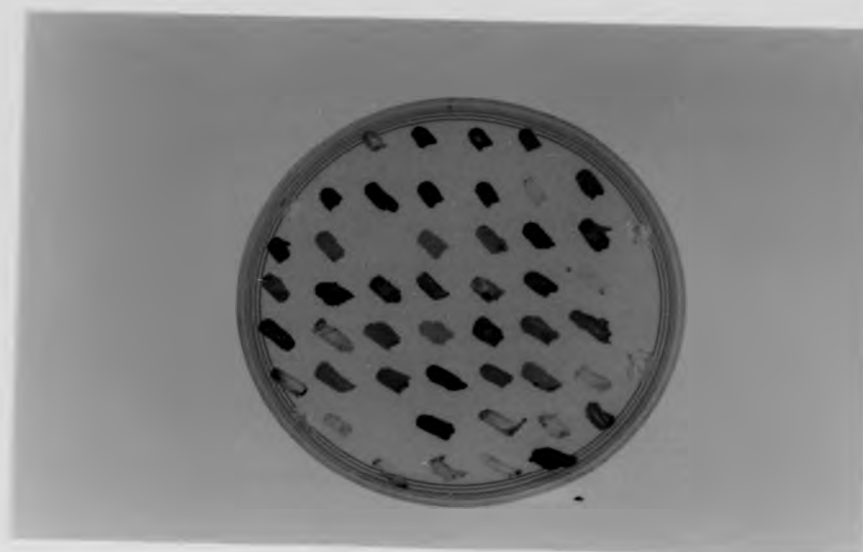


Figure 5.4.1 A selection of pigment mutants isolated after UV-mutagenesis of RB4.

Colonies with altered pigmentation (section 5.2.3) were repatched on fresh plates and grown under microaerophilic conditions.

types (eg 'grey' mutants) was enhanced by use of the microaerophilic growth conditions. Some mutants were shown to exhibit enhanced autofluorescence using the method of Youvan et al (1983), and this method could be used to isolate mutants with subtle alterations in the photosynthetic apparatus. The analysis of the pigment mutants obtained is described in Chapter 6. The ease of isolation of pigment mutants, and therefore membrane-development and electron transport mutants, greatly increases the potential for studying those processes in Rm. vannielii. This is an exciting prospect since this organism has some unique characteristics in terms of the photosynthetic apparatus and its development during the swarmer cell cycle (Kelly, 1985; Kelly and Dow, 1985).

5.4.2 Temperature-sensitive mutants

The most obvious way forward in the genetic analysis of the differentiation of the swarmer cell is the isolation and analysis of temperature-sensitive (ts) mutants. The swarmer cell represents an obligate stage in the developmental cycle and must therefore be coded for by essential genes. It follows that any mutation in such functions would be lethal unless conditional mutants are isolated. Prior to the development of the microaerophilic growth technique the isolation of ts-mutants would have been technically very difficult as the temperature in gas bags is difficult to control. However, a system using tubes was developed. This involved picking colonies and inoculating each into two pipettes filled with solid medium. Each tube was overlaid with wax and they were incubated in separate tanks, one at 25°C and the other at 35°C, under tungsten lamps. This system was very labour-intensive especially as it is usually necessary to screen at least 1000 colonies

to obtain some classes of mutants. Nevertheless the system could be useful in the isolation of ts-mutants in strains unable to grow in the dark.

The microaerophilic incubation technique gives the ability to control temperature precisely and work with plates. It is therefore feasible to isolate ts-mutants more easily. In one UV mutagenesis experiment, RB1 (Rm5, chain-forming and Sm-resistant) colonies were initially grown up at 25°C and then patched onto plates and regrown at 25 and 35°C. 3500 colonies were screened and one ts-mutant was isolated. This was a very poor yield. However, in the same experiment pigment mutants were very rare ($<0.01\%$) suggesting inefficient mutagenesis. The morphology of the ts-mutant isolated appeared normal and did not change when subjected to the non-permissive temperature. The isolation of this mutant demonstrates that, with more efficient mutagenesis and extensive screening of colonies, it should be possible to isolate a range of conditional-lethal mutants specific for the swarmer cell cycle. This is a most exciting prospect in terms of the analysis of the developmental biology of Rm. vannieli.

5.4.3 Motility mutants

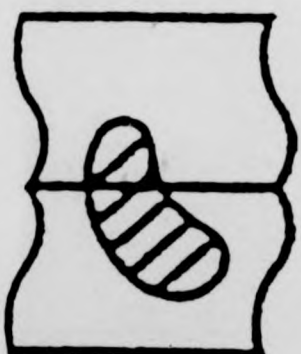
A major morphological difference between swarmer cells and chain/stalked cells is the possession of peritrichous flagella and the shedding of these structures is a major landmark event in the swarmer cell cycle. The isolation of motility mutants could therefore be useful in differentiation studies, and in the study of tactic responses in Rm. vannieli. In the same UV mutagenesis experiment mentioned above, the colonies were screened for motility/non-motility at 25 and 35°C.

Screening involved stabbing a toothpick carrying the inoculum into sloppy agar (0.3% bacto agar) plates. As a result 27 mutants, 0.77% of the total screened, were isolated and these fell into three classes: not motile at any temperature; cold-sensitive for motility (ie were motile at 35°C but non-motile at 25°C); and temperature-sensitive for motility.

Figure 5.4.3a shows the appearance of such mutants compared to wild type in sloppy agar. The wild type forms large haloes of growth around the central inoculum but in non-motile mutants, or the non-motile 'pairs and swarmers' strain, growth is restricted to the point of inoculation. In addition, variation in halo type was observed in that some haloes were uniform and others had a speckled appearance with numerous red dots in the swarm region. The batch of non-motility mutants was subjected to preliminary analysis using optical and electron microscopy. In brief they all formed swarmer or single cells and 2 out of 15 showed signs of normal motility under the light microscope, 4 showed the ability to 'tumble' with the inability to move in straight lines. In addition some of the non-motile isolates appeared to have much longer prosthecae than the wild type controls grown under the same conditions (Figure 5.4.3b). A similar observation was made in Tn5-induced motility mutants of Rm. vannielii (MacDonald, 1988). Electron microscopy showed that some of the non-motile isolates had flagella, whilst in others no flagella were present.

The isolation of conditional mutants for motility is further evidence that it should be possible to isolate conditional-lethal mutants affected in cell cycle functions. The availability of motility mutants should facilitate further analysis of the shedding of flagella by swarmer cells during differentiation and the investigation of

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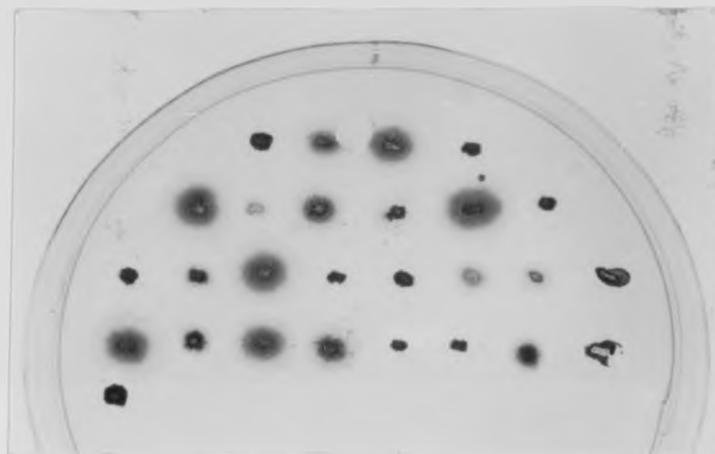
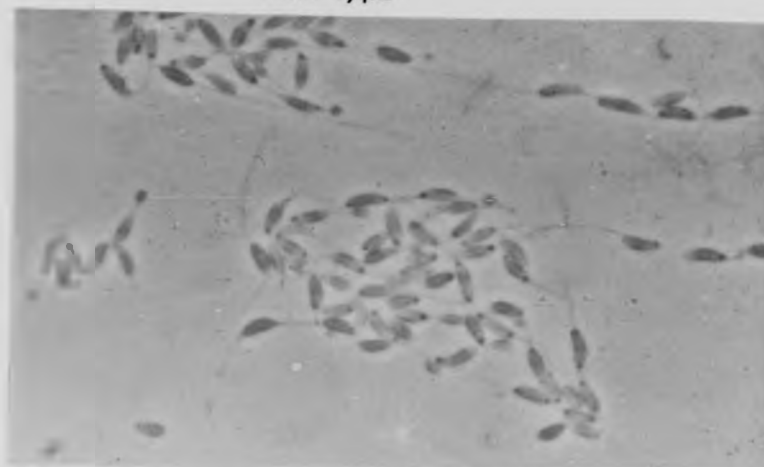


Figure 5.4.3a Assay for motility mutants

Motility mutants of RB1 (Rm5, chain forming and Sm^R) isolated after UV mutagenesis. Colonies were picked and stabbed into PYM sloppy agar.

wild type

175



mutant

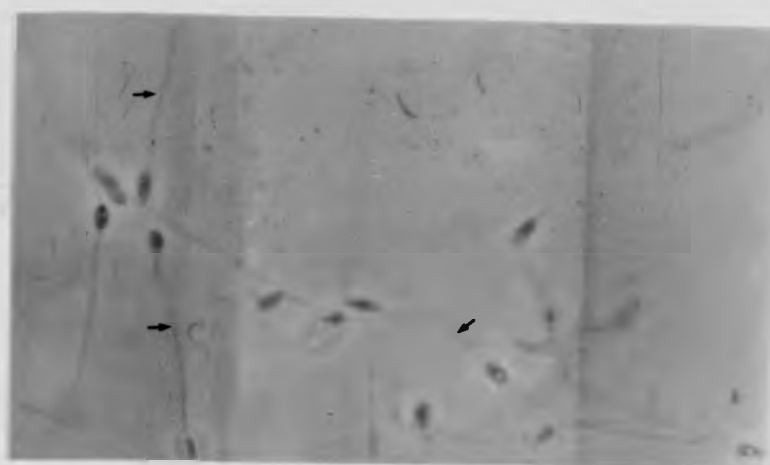


Figure 5.4.3b Abnormally long prosthecae exhibited by some non-motile mutants

chemotaxis/phototaxis in this organism.

5.4.4 Morphological mutants

A lot of variation in colony morphology was observed after mutagenesis and this was not investigated further. However, colony morphology can reflect changes in cell structure, ie chain-forming strains give rise to rough, irregular, dry colonies whereas 'pairs and swarmers' strains form round, smooth, shiny colonies (Dow and France, 1980). In agar tubes, chain-forming strains form irregular, roughly spherical colonies with 'rosette' patterns on the surface; 'pairs and swarmers' strains form regular trilobate colonies (Figure 5.4.4). This difference may reflect the inability of the latter strain to form chains of cells or alternatively the non-motile nature of such strains. As mentioned above some revertants to a chain-forming phenotype were observed after UV mutagenesis of RB4. Thus, analysis of colonies showing aberrant morphology could yield some interesting mutants of cell type-expression, or structural mutants.

5.4.5 Auxotrophs

Attempts to isolate auxotrophs by screening colonies for the ability to grow on PYM (0.1% (w/v) YE) and inability to grow on PM without supplements yielded no mutants. It was suspected that this may reflect an impermeability to certain growth factors. Subsequent to the observation that Rm. vanniellii could grow on media containing 0.8/1.2% (w/v) YE depending on strain (Chapter 4), a screen of 500 colonies for auxotrophy was performed on medium containing 0.6% (w/v) YE. Two putative auxotrophs were isolated, exhibiting growth on PYM (0.6% (w/v)

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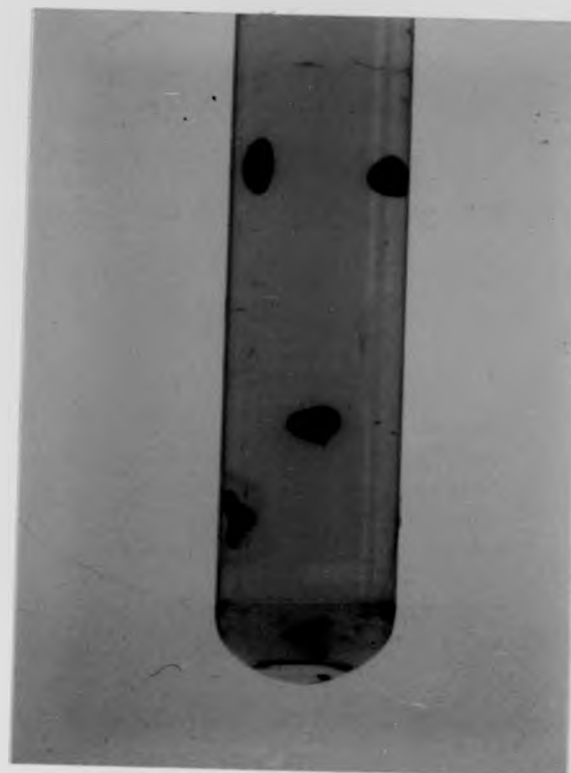


Figure 5.4.4 Three dimensional, tri-lobate colonies formed in agar columns inoculated with the 'pairs and swimmers' strain of Rm.vannielii

YE) and no growth on PM. These mutants were not analysed further.

5.5 Conclusions

This work demonstrates that both NTG and UV mutagenesis may be used for Rm. vanniellii, and have given high yields of mutants. The pigment mutants are analysed further in the next chapter. Again the microaerophilic growth technique was found to be superior to the 'gas-bag' incubation technique in mutagenesis experiments.

3.2 Conclusions

This work demonstrates that both the
the results, and have given a
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microchemical growth technique in
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CHAPTER 6

CHARACTERIZATION OF PIGMENT MUTANTS

6.1 Introduction

The photosynthetic apparatus of Rm. vannielii has a lamellate intracytoplasmic membrane system (ICM) (Conti and Hirsch, 1965), which carries the photosynthetic pigments (ie bacteriochlorophyll (Bchl) and carotenoids) in pigment-protein complexes. These complexes have recently been characterized (Kelly, 1985; Kelly and Dow, 1985). There are two pigmented complexes containing different spectral forms of Bchl-a. LHII, the 'accessory' light-harvesting complex (90kD) contains light-harvesting Bchl and two polypeptides (11 and 13kD). This complex is designated B800-865 due to its in vivo absorption maxima. The second complex (150kD) contains the primary light-harvesting complex (LHI) and the photochemical reaction centre (RC), and is designated B885-RC. LHI contains light-harvesting Bchl and two polypeptides of 12 and 14kD. The RC contains RC-Bchl and RC-bacteriopheophytin and three polypeptides of 26, 28 and 31kD. A membrane-bound cytochrome, C-553, is associated with the 31kD subunit of the RC (Kelly, 1985; Kelly and Dow, 1985). The components of the two pigment-protein complexes are illustrated in Figure 6.1.

Both complexes (B800-865 and B885-RC) contain carotenoids giving absorption maxima at wavelengths 460, 490 and 525nm. Several papers have been published on the carotenoid content on Rm. vannielii (Volk and Pennington, 1950; Conti and Benedict, 1962; Ryvarden and Laaen-Jensen, 1964; Britton, 1975). All eight intermediates of the normal spirilloxanthin series have been identified, and the major ones found under anaerobic light conditions are lycopene (5%), rhodopin (64.6%), anhydrorhodovibrin (6.6%) and spirilloxanthin (21.6%), (% total carotenoid) (Britton et al. 1975). In addition, the presence of small

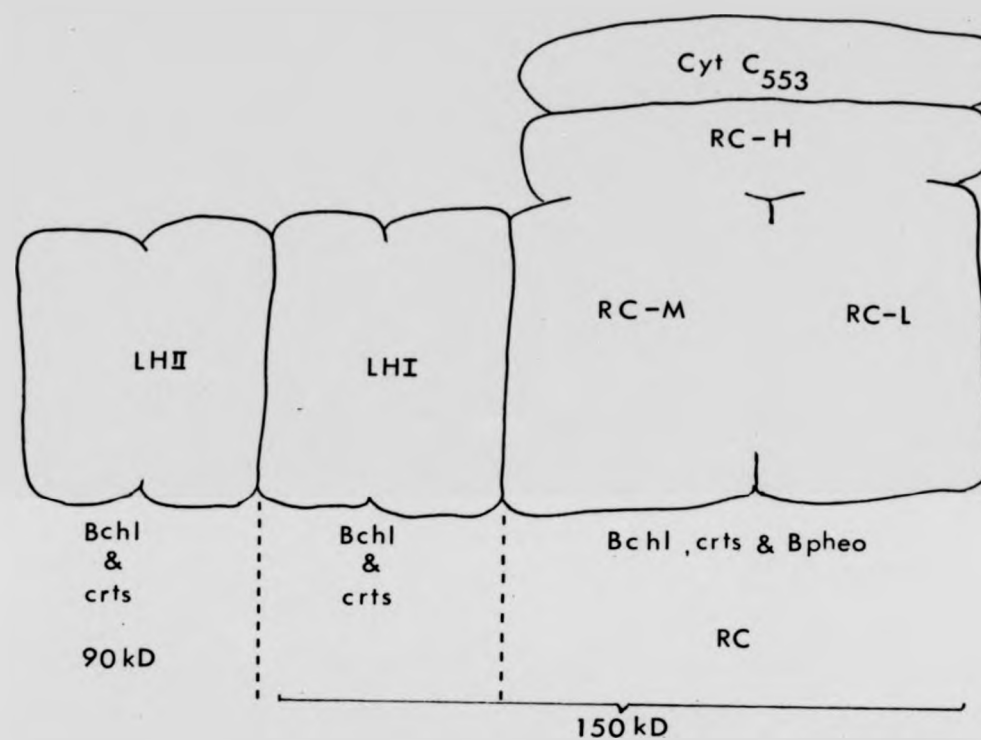


Figure 6.1 Composition of the pigment-protein complexes of the ICM's of *Rm. vannielii* (Kelly, 1985; Kelly and Dow, 1985a)

amounts of mono and dimethoxy derivatives of lycopene indicated a possible series of overlapping or interrelated biochemical pathways (yet to be elucidated). Further complications arise due to the presence of β -carotene and two other cyclic carotenoids (β -cryptoxanthin and 1'-methoxy-3'-4' didehydro-1'-2' dihydro- β -carotene).

A large number of pigment mutants were isolated during this work. This chapter describes the characterization of a selection of those mutants in an attempt to identify the possible biochemical lesions in each of the mutants.

6.2 Absorption spectra of cell-free extracts of mutants

6.2.1 Absorption spectrum of the wild-type

Absorption spectra of cell-free extracts were measured as detailed in Section 2.38. The spectrum for wild-type cell-free extracts, (Figure 6.2.1) had the following characteristics: (1) absorption maxima at 378/380 and 595nm due to mature Bchl-a (in other organisms the position of these peaks is known not to be affected by Bchl-a protein interaction); (2) 2 maxima at 800 and 872nm due to the Bchl-a-protein complexes.

Purified LHI from *Rm. vanniellii* absorbs at 885nm (Kelly, 1985), but when LHII is present in the photosynthetic membrane, a composite band occurs at approximately 872nm. The absorption band at 800nm was predominantly due to the LHII complex with a lesser contribution from RC. In the absence of LHII the RC gave peaks at 760nm due to bacteriopheophytin and 800nm due to RC-Bchl-a; (3) a triplet of maxima at 460, 490 and 525nm due to the carotenoid pigments. Finally, absorption spectra of purified

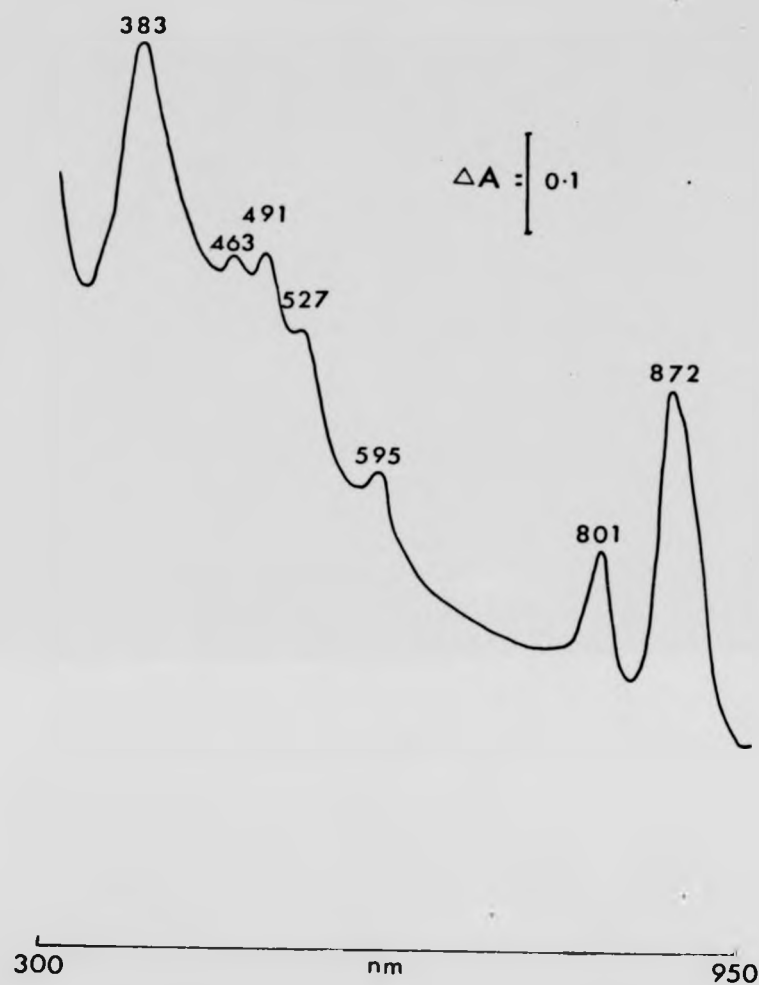


Figure 6.2.1 Room temperature absorption spectrum of wild-type cell-free extracts of *Rm.vannielii* RB4 grown under microaerophilic (1% O₂) dark conditions

ICM exhibited an additional peak in the 415-420nm region. This peak was not associated with the pigment-protein complexes, and Kelly (1985) suggested that this peak was due to flavoprotein. A yellow pigmented fraction was observed at the top of sucrose gradients after centrifugation to isolate pigment-protein complexes. The absorption spectrum of this fraction had a single major peak at 418nm.

6.2.2 Spectra of mutants

The mutants were classified into 4 major groups according to the appearance of their absorption spectra (Table 6.2.2).

The mutants of the first group were altered only in the carotenoid region of the spectrum. Generally, the 527nm peak was reduced or absent, with residual bands at 460 and 492nm and a shoulder occurred at 412/416 in one third of the mutants in this class. These mutants were orange/brown in colour and a representative spectrum is shown in Figure 6.2.2a(1).

The second group of mutants was generally lacking the 460 and 490nm carotenoid bands with a residual band or shoulder at about 527nm. In addition the 872nm maximum was shifted to about 884nm and the 800nm maximum was reduced in magnitude with a shoulder at 760nm. These mutants were grey in colour and a typical spectrum is shown in Figure 6.2.2a(2). A subgroup of 2 mutants were grey/brown in colour and had a large absorption peak at 525nm. In the IR region both had a much broader than normal 800nm peak, with shoulders at 740 and 760nm. In one case the 872nm maximum was shifted to 880nm (Figure 6.2.2b(1)), and in the other it was greatly reduced with a doublet of peaks in the 880nm region

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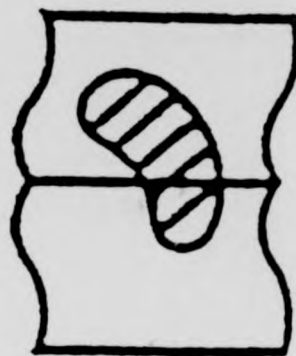


Table 6.2.2 Classification of pigment mutants according to absorption spectra

Strain	Mature Bchl-a	Putative Bchl-a Precursors	Flavo-protein	C A R O T E N O I D S	Bacterio-pheophytin	LHII/RC Composite	LHI/LHII Composite	LHI alone	Group colour		
RB4	383/595	-	masked*	460	490-2	525-7	masked	800-4	872-6	880-4*	Wild type
UV17	372sh/596	700	412sh	460	490sh			804	876		
UV22	380/596	672	416sh	460	490sh	530sh		804	876		Group1
NTG42	383/595			458	491red			801	872		Orange
NTG58	379/595			458	491			800	872		Brown
UV47	383/595			463	491	572red		800	872		
UV40	377/595					530red	760	801	884		Group2
NTG77	383/595					527red	760	801	884		Grey
NTG37	383/595				486	518red	760	801	884		
NTG16	383/595					527red	760	801	884		
UV32	380/595				490sh	525		803	880		Sub-group
NTG29	380/595					525	748	803	880red		Grey/Brown
UV36		421/669									Group3
NTG52		385/667									
NTG9		667	413	455							
UV10		382/668	415	455							
UV46		360/590	424								
		630/652									
NTG43		372	415	455							Group4
UV12			413	460							
UV15			418	460							
NTG78		372	415	455							Yellow
NTG67		380	415	455							

* from Kelly, 1985; sh = shoulder; red = reduced peak

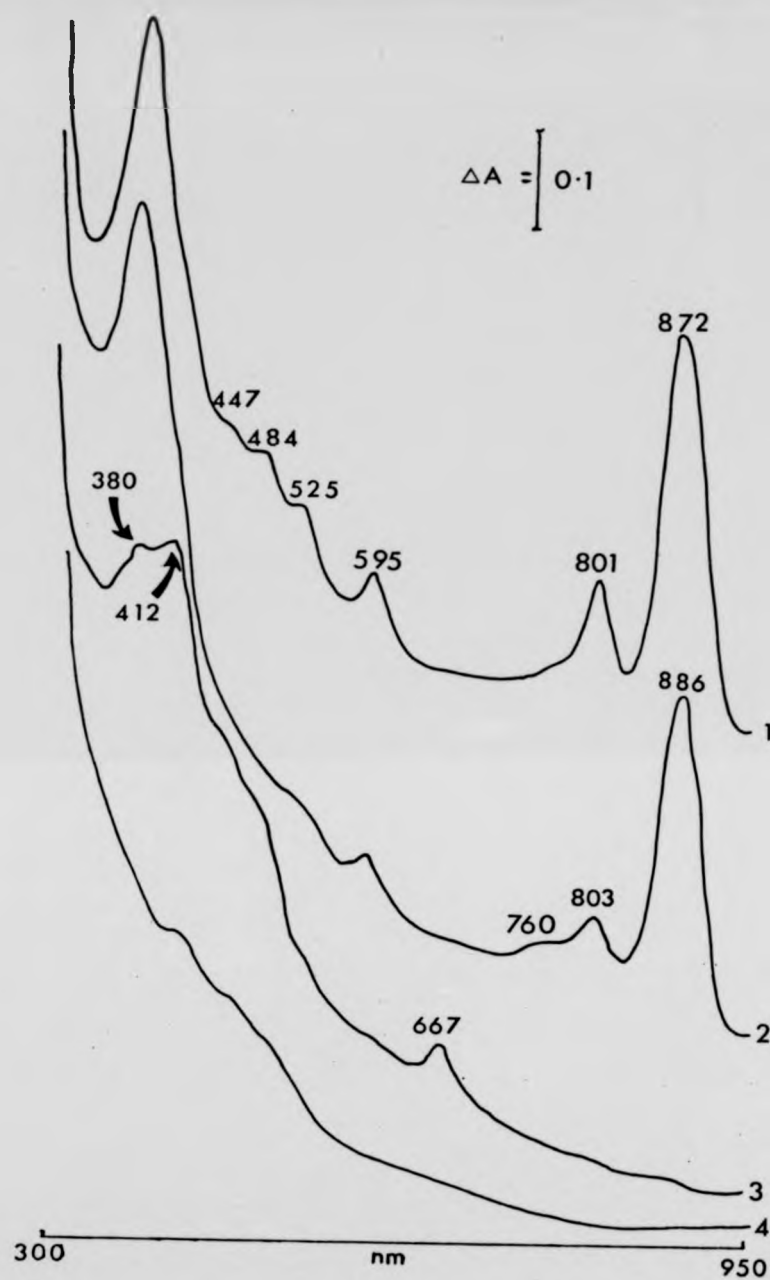


Figure 6.2.2a Absorbance spectra of pigment mutants

Room temperature absorbance spectra of cell free extracts from representatives of each mutant group: (1) brown/orange; (2) grey; (3) green (4) yellow. All strains were grown under microaerophilic/dark conditions.

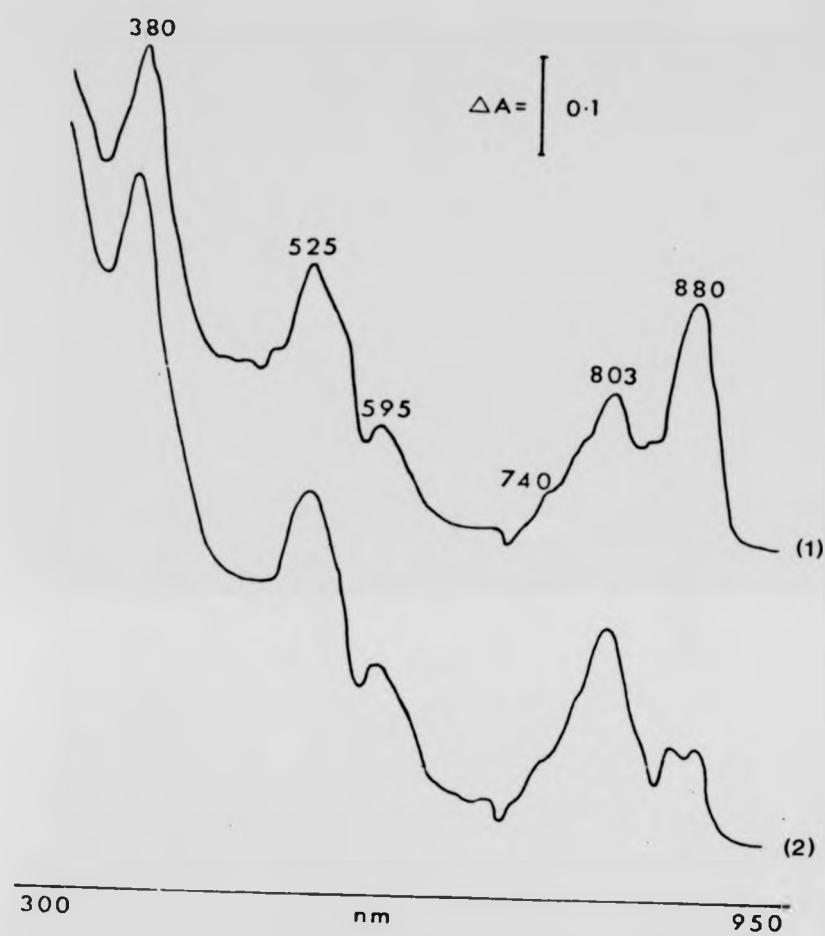


Figure 6.2.2b Absorption spectra of grey/brown mutants.

(Figure 6.2.2b(2)).

The third group of mutants had Bchl-a-like absorption bands at 380 and 667nm. Some of these mutants (2 out of 7) had greatly reduced maxima at 800 and 872nm normally associated with pigment-protein complexes, but generally these bands were absent. That mature Bchl-a bands (383/595nm) were either absent or represented by slight shoulders. In the carotenoid region, the 527nm band was always absent, and the 460/490nm bands were either absent or reduced to slight shoulders. In most cases, a band at 416nm corresponding to that observed in the first group was found. These mutants were green in colour and a representative spectrum is shown in Figure 6.2.2a(3).

The fourth group of mutants had bands in the 370-380nm region, but otherwise all the bands associated with mature Bchl and its inclusion in pigment-protein complexes were absent. In the carotenoid region they all had a shoulder at 460nm and the 490/527nm bands were absent or represented by very slight shoulders. In all cases, a shoulder/band was observed in the 416nm region. These mutants were yellow in colour and a representative spectrum is shown in Figure 6.2.2a(4).

6.3 Analysis of pigment-protein complexes of mutants by semi-denaturing PAGE

A selection of mutants were analyzed on PAGE under semi-denaturing conditions to compare their pigment-protein complexes with those of the wild-type, and to correlate the spectral alterations with any changes in protein profile (the components of the photosynthetic apparatus are summarized in Figure 6.3a). The wild-type membrane profile is shown in

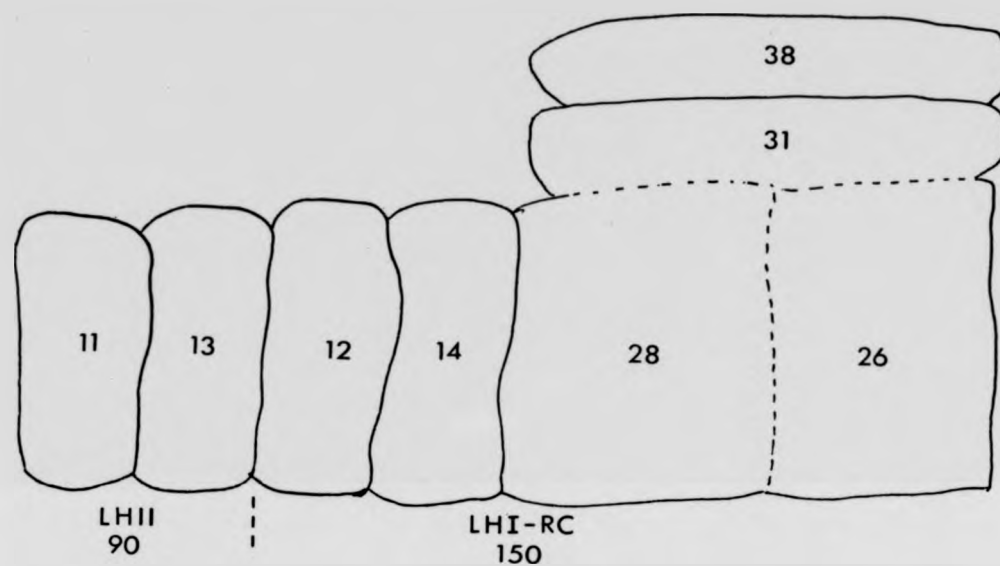


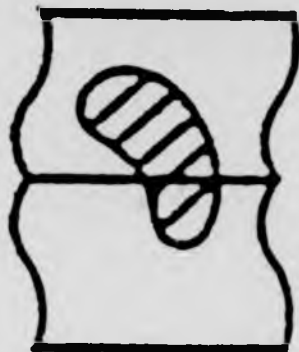
Figure 6.3a Polypeptide components of the pigment-protein complexes from the ICM's of *Rm. vannielii* (Kelly, 1985) M_w -kD

Figure 6.3b. This system of analysis of the ICM of Rm. vannielii was developed by Kelly (1985), and it demonstrates the presence of two pigment-protein complexes: LHI-RC, 150kD and LHII, 90kD. This gel system, using SDS and Triton X-100 to give stable semi-denaturing conditions, is designed to remove pigment-protein complexes from the membrane in aggregated form. Kelly found that the spectral analyses of the pigmented bands obtained correlated well with the in vivo absorption spectra suggesting that the organization of the pigment-protein complexes had not been significantly disrupted in the process. Kelly suggested that conclusions about the in vivo organization of the photosynthetic apparatus based on such gel patterns must take account of the possibility of micellar artefacts. Such artefacts could be more apparent in mutants with altered ICM structures. In this work, 7.5% (w/v) gels were used to give a better separation of these high molecular weight complexes. As a result extra bands were obtained in association with the LHI-RC complex (Figure 6.3b). These extra bands in the 150kD region probably represent aggregates of varying size formed as a result of oligomerization during electrophoresis. Nevertheless, the method is still suitable for the purpose of showing the status of the pigment-protein complexes compared to the wild-type.

Figure 6.3c shows the patterns obtained when the ICM from a selection of mutants were subjected to such analysis. The orange/brown mutants (Group 1, UV17 and UV22) had both of the wild-type pigment-protein complexes at 90kD and 150kD and the aggregates associated with the LHI/RC complex in the 150kD region. In addition, extra non wild-type bands (eg a, e) were observed in the 90-150kD region.

The grey mutants (Group 2, UV32, NTG29 and NTG62) chosen for this

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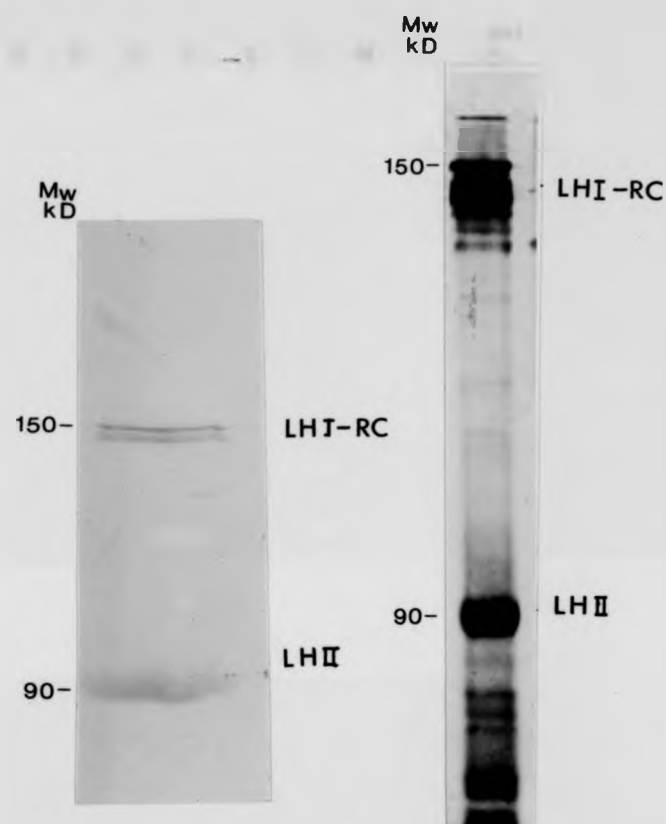
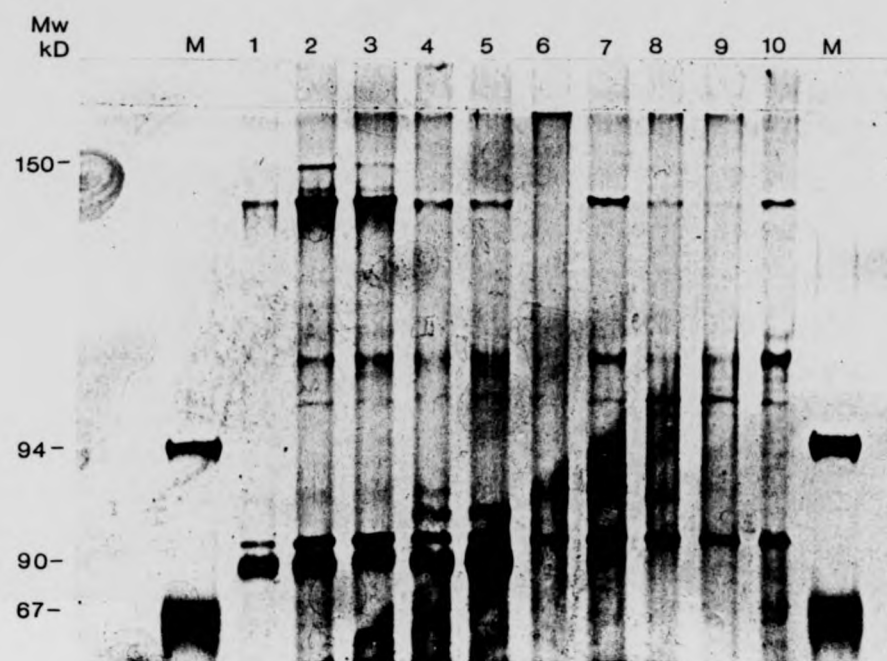


Figure 6.3b Intra-cytoplasmic membrane protein profile of *Rm. vannielii* RB4; PAGE with semi-denaturing conditions was used to separate pigment protein complexes.

ICM samples prepared from microaerophilically grown cells were solubilized at RT by mixing with an equal volume of 1.6% (w/v) SDS and 1.6% (w/v) Triton X-100 in 61 mM Tris-HCl pH 6.8 buffer. After centrifugation supernatants were run on the Triton-SDS gel system (Section 2.22.3). Acrylamide concentration, 7.5% (w/v). Loading - 150 μ g protein/track. The gel was photographed unstained (left) and stained using silver stain (right).



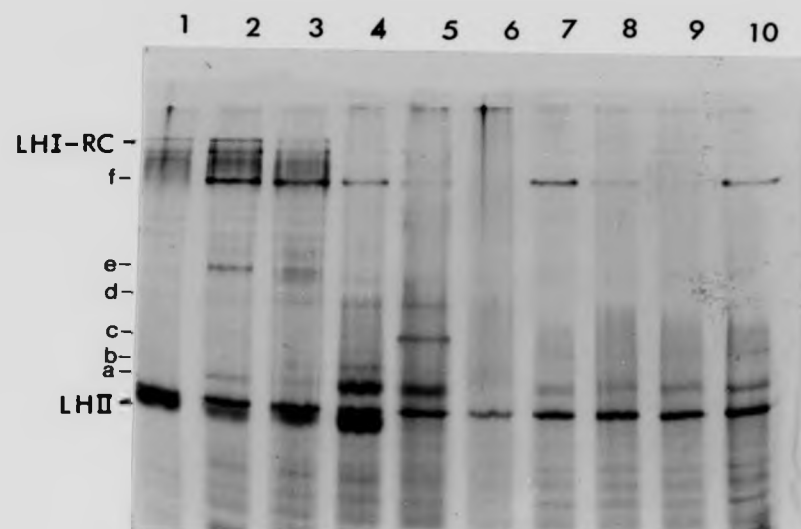


Figure 6.3c Intra-cytoplasmic membrane protein profiles of a selection of pigment mutants compared to the wild type profile

Track	Strain	Group
1	RB4	Wild type
2 and 3	UV17 and UV22 respectively	Brown/Orange/Gp1
4 and 5	UV32 and NTG29 respectively	Grey/Brown/Gp2
6	NTG62	Grey/Gp2
7 and 9	UV36 and NTG52 respectively	Green/Gp3
8 and 10	NTG43 and UV12 respectively	Yellow/Gp4
M	Markers	

Method, conditions and staining were as described for Figure 6.3b; loading 50 μ g protein/track

analysis were found to be atypical of the group as indicated by spectral analysis. UV32 had no LHI/RC band at 150kD but had extra bands in the 90-150kD region: the one of least mobility co-migrating with extra band (a) observed in the orange/brown mutants. However, in this mutant it was present in a much higher concentration. UV32 appeared to have a normal LHII band running at 90kD. NTG29 had neither of the wild-type LHI/RC or LHII bands at 150 and 90kD respectively, but instead had two extra bands (a, c) in the 90-150kD region. Again, the one of lower Mr ran at the same position as the extra band observed in UV32 and the two orange/brown mutants. This species was present in a higher concentration than observed in the orange/brown mutants. NTG62 was deficient in both LHII and LHI/RC and also appeared to be lacking most of the other high Mr proteins, except for one (just above 90kD).

The four green/yellow mutants analyzed (Group 3 UV36, NTG52; Group 4, NTG43, UV12) were all very similar in their protein profiles, lacking both LHI/RC and LHII bands. They all exhibited the extra band (a) just above 90kD, as observed in the other mutants. In addition NTG52 appeared to lack a band (f) at about 120kD that was present in all other samples except NTG62.

6.4 Polypeptide composition of ICM's from mutants

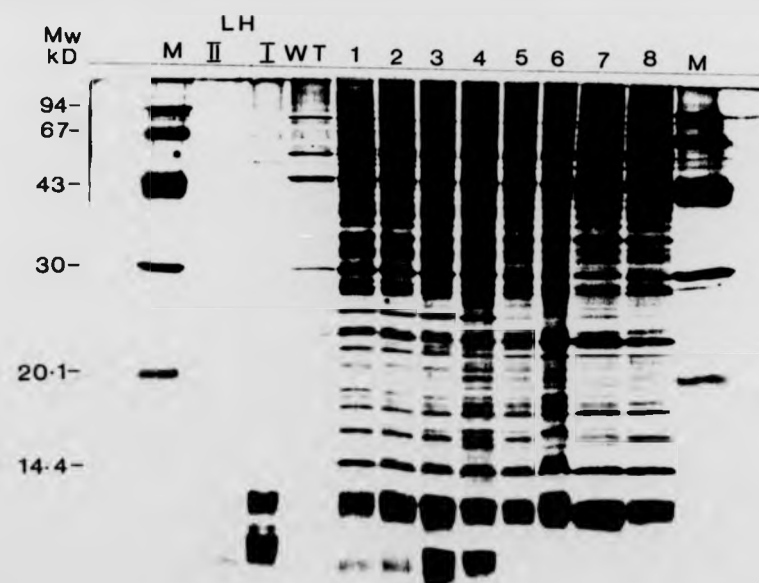
The polypeptide content of the membranes from selected mutants is shown in Figure 6.4. The orange/brown mutants (Group 1, UV17 and UV22) contained all polypeptides as found in the wild-type. The three RC polypeptides (26, 28 and 31kD), the RC-associated cytochrome C-553 (38kD) and the four light-harvesting polypeptides (LHI, 12 and 14kD; LHII, 11 and 13kD). The grey mutants (Group 2, UV32 and NTG29) exhibited

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Figure 6.4 (cont'd)

Tracks	Strains	Group
LHII	LHII purified from wild type	
LHI	LHI purified from wild type	
1 and 2	UV17 and UV22 respectively	Orange/Brown GP1
3 and 4	UV32 and NTG2a respectively	Grey/Brown GP2
5 and 7	UV36 and NTG52 respectively	Green Gp3
6 and 8	NTG43 and UV12 respectively	Yellow Gp4
WT	Wild Type	



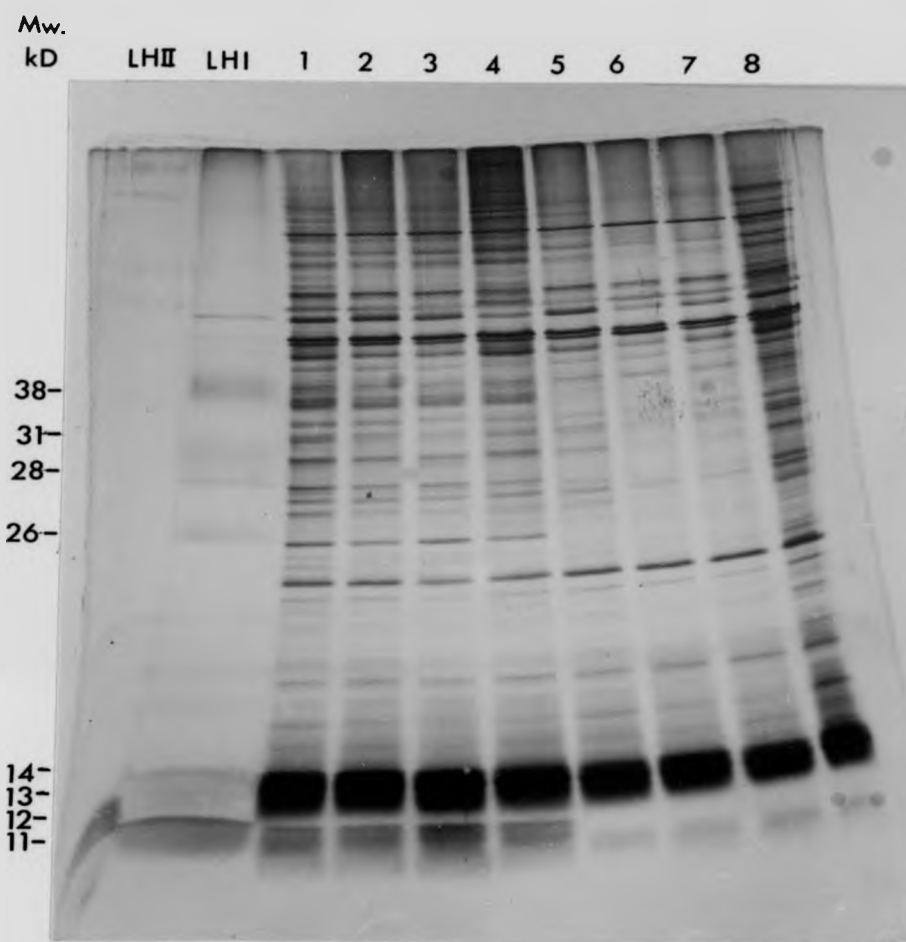


Figure 6.4 Intra-cytoplasmic membrane polypeptide of pigment mutants

ICM preparations (15 μ g protein/20 μ l sample) were denatured in Laemmli sample buffer for 2 min at 75°C and electrophoresed on a 10-30% w/v gradient gel (20 mA, cold O/N) which was subsequently silver-stained. Identity of bands and molecular weights are known from other gels (opp. page) in a series using these ICM prep's, purified pigment-protein complexes and Pharmacia molecular weight markers.

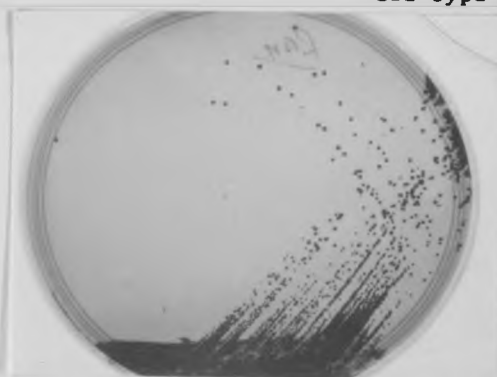
the same polypeptide content as the wild-type (it is possible that they were missing the 31kD RC polypeptide (H); however this band is usually very faint).

The four green/yellow mutants all showed a common protein profile: they were deficient in the RC polypeptides (26, 28 and 31kD) and the associated C-553 cytochrome (38kD), and had all of the low molecular weight pigment-binding polypeptides, except for the 12kD polypeptide from LHI which was absent in all four cases.

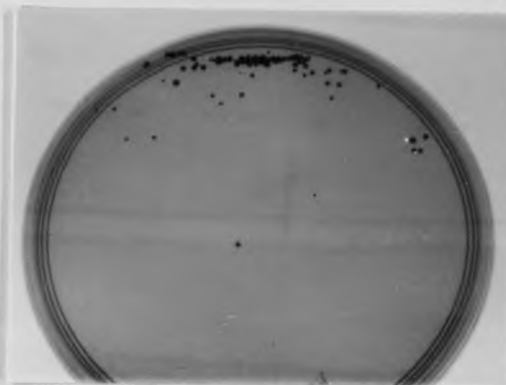
6.5 Measurement of enhanced autofluorescence of grey mutants UV32 and NTG29

As discussed later, the PAGE analysis and spectral analysis of UV32 and NTG29 suggested that these mutants exhibited alteration in the structure of their pigment-protein complexes. The spectra showed that there were carotenoid pigments and light-harvesting Bchl present. It follows that some harvesting of visible light should be possible, and if the pigment-protein complexes are abnormal then the photochemical energy transfer through such complexes would be less efficient than in the wild-type. In such altered systems, the inefficient transfer of energy through photochemical pathways results in light being re-emitted causing enhanced auto-fluorescence.

Using an apparatus similar to that described by Youvan et al (1983), fluorescence from UV32 and NTG29 were compared to two wild-type strains, Rm5 and RB4. Figure 6.5 shows the results of high-speed infra-red photography used to assess the level of fluorescence in the near infra-red region. Both UV32 and NTG29 showed enhanced fluorescence as compared



Mutant NTG29



Mutant UV32

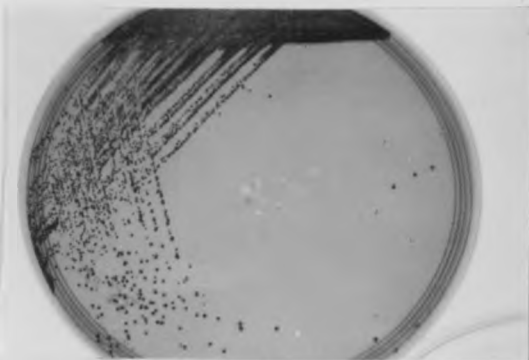


Figure 6.5 Enhanced autofluorescence of pigment mutants

Colonies were grown on PYM agar under microaerophilic conditions. Plates were photographed in the absorbance mode (left) (normal photography) to record location of colonies and then for 3 min in the fluorescence mode (right) as described in section 2.17.

to the wild-type strains.

6.6 Discussion

6.6.1 Orange/brown mutants (Group 1)

The Rhodospirillaceae synthesize coloured carotenoids belonging to the 'spirilloxanthin series'. In Rb. capsulatus and Rb. sphaeroides, spheroidene and spheroidenone predominate under anaerobic conditions in the light. Rs. rubrum synthesizes mainly spirilloxanthin. In R. acidophila rhodopin, rhodopinal and rhodopinal-d-glucose are the predominant pigments, as are spirilloxanthin, lycopene and rhodopin in R. palustris. The different species contain different carotenoid series and the intermediates which predominate vary from species to species, and with growth conditions. Thus it is not possible to make direct detailed comparisons between mutants obtained here and carotenoid-altered mutants from other species. Due to the complex carotenoid content of Rm. vannielii it is not possible to make any identification of the intermediates accumulating in brown/orange mutants on the basis of in vivo spectra. Further work (eg TLC or HPLC analysis) to isolate and identify the pigments accumulated in the mutants was not carried out.

This group of mutants had spectra which were altered in the carotenoid region of the spectrum only. Similar mutants have been described in other species. Green mutants, presumably blocked at the neurosporene or cloroxanthin stages of carotenoid biosynthesis, have been obtained from Rb. sphaeroides (Griffiths and Stanier 1956; Connelly et al., 1973; Saunders and Jones, 1974), and for Rb. capsulatus (Weaver et al., 1975;

Yen and Marrs, 1976). Brown mutants have been isolated for Rb. sphaeroides (Griffiths and Stanier, 1956; Segen and Gibson, 1971). Yen and Marrs (1976) isolated yellow mutants Rb. capsulatus which were phenotypically similar to the brown mutants observed in Rb. sphaeroides by Griffiths and Stanier (1956). These mutants are all phenotypically similar to the brown/orange class observed here in that they are altered only in the carotenoid region of the spectrum. The different colours observed are due to the differences in normal carotenoid content as explained above.

The spectra of the orange/brown mutants were identical to those of the wild-type in respect of the near-IR maxima and the Bchl-a maxima at 378 and 595nm. This suggests that mature Bchl-a was present and that they contained normal pigment-protein complexes in respect of Bchl-a.

Analysis on semi-denaturing PAGE suggested the presence of normal LHI/RC and LHII complexes. However, additional bands were observed in the 90-150kD region of the gel and these may indicate that alteration in the carotenoid content of the pigment-protein complexes could possibly lead to changes in the structure of the ICM. Alternatively, the additional bands could be due to changes in the ICM system apart from the photosynthetic apparatus caused by secondary mutations. The latter is less likely since similar alterations were observed in two different mutants. Analysis of the ICMs from the two orange/brown mutants suggested the presence of all the normal polypeptide components of the photosynthetic apparatus. This could be verified using antibodies raised against LH-polypeptides from other species. The mutants therefore have all the necessary components of the photosynthetic apparatus, ie Bchl-a and the normal light-harvesting and RC polypeptides assembled in apparently normal pigment-protein complexes in the ICM. It would be

interesting to compare the growth rates of these mutants under a range of light intensities with those of the wild-type since one of the functions of carotenoid pigments is the harvesting of light in the visible region of the spectrum.

6.6.2 Grey mutants (Group 2)

This group of mutants was very similar to the classical blue/green mutants isolated in other members of the Rhodospirillaceae in that the near absence of coloured carotenoids is associated with shifts in the peaks due to Bchl-protein interactions in the IR region of the spectrum. The shift of the 872nm peak to 884nm and the reduction in magnitude of the 800nm peak with a shoulder at 760nm can be interpreted as being due to the absence of the LHII complex. The LHI complex alone absorbs at 885nm, Kelly (1985), but when the LHII is present in the photosynthetic membrane a composite band occurs at 872nm. The absorption band at 800nm in the wild-type is mostly due to the LHII complex with a lesser contribution from the RC. Removal of LHII would therefore cause a reduction in the size of the 800nm peak and the unmasking of a shoulder at 760nm due to RC- bacteriopheophytin. Blue/green mutants lacking coloured carotenoids and accumulating phytoene, have been described for Rb. sphaeroides (Griffiths and Stanier, 1956; Griffiths et al. 1955; Sistrom et al. 1956; Clayton and Smith, 1960; Sistrom and Clayton, 1964; Sistrom, 1966) for Rb. capsulatus (Drews et al. 1971; Weaver et al. 1975; Yen and Marrs, 1976) and for Rs. rubrum (Cuendet and Zuber, 1977). The in situ Bchl absorption spectra of blue/green mutants of Rb. sphaeroides and Rb. capsulatus differ from the wild-type in a fashion similar to that described above, and certain polypeptides found in wild-type membranes are missing from blue/green mutants. These changes have

been described as a loss of the light-harvesting Bchl-(LHII). Carotenoid-minus, LHII-plus strains have never been found. This suggests that there is a connection between the appearance of coloured carotenoids and LHII-Bchl. It has been suggested that the LHII absorption spectrum is caused by a Bchl-carotenoid conjugate and thus the loss of carotenoids must always lead to a loss of LHII (Weaver et al. 1975). Marrs (1978) speculated on an alternative explanation: carotenoidless mutants were unable to grow photosynthetically unless they lacked LHII; thus whenever viable, blue/green, photosynthetically competent mutants were isolated they would necessarily contain a second mutation blocking LHII formation.

Genetic analysis of blue/green mutants of Rb. capsulatus suggested that the separate mutations were involved in the blue/green phenotype. Blue/green cells of Rb. capsulatus were crossed with GTA from wild-type cells. Many cells that regained the ability to synthesize carotenoids still had altered Bchl-IR absorption spectra, indicating that a second mutation was present in the blue/green mutants which caused the Bchl spectral alterations (Drews et al. 1976; Marrs, 1978).

Other evidence supporting this conclusion includes the fact that phenocopies of the carotenoidless strains of both Rp. gelatinosa (Crounse et al. 1963) and Rs. rubrum (Nugent and Fuller, 1967) have normal Bchl spectra. A further mutant strain of Rb. sphaeroides was isolated by Segen and Gibson (1971). Under semi-aerobic conditions this strain is brown rather than red (as in the wild-type). It appeared to accumulate the normal carotenoid spheroidene, but was unable to oxygenate this pigment to spherodenone, which gives semi-aerobic cultures of the wild-type their red colour. Despite the presence of

spheroidene, the spectrum of Bchl in this strain was similar to that of strains lacking coloured carotenoids, ie it had altered Bchl peaks in the IR region.

Thus there is a lot of speculation in the literature as to the cause of the spectral alterations observed in the classical blue/green phenotype. Although the evidence above suggests that two mutations are required, some authors (eg Sistrom et al., 1956) claim that disruption of carotenoid biosynthesis and alteration in the spectrum of Bchl can result from a single mutation. This conclusion is based on the fact that these effects are correlated in independent isolates in several different species.

The mutants isolated here suggest that Rm. vanniellii could be added to that list of species, but further work would have to be carried out to prove whether the phenotype was due to more than one mutation or a pleiotropic effect due to a single mutation. A simple approach would be to perform selection for reversion and see if any of the revertants were restored to wild-type carotenoid content but retained the alterations in the IR Bchl peaks. Alternatively, complementation analysis using a broad host-range vector (Chapter 8) could resolve the problem in a fashion similar to that used by Marrs (1978) with GTA mapping.

The two grey/brown mutants have spectra which were very different from the classical blue/green phenotype. Both had major peaks at 525nm whereas the rest of the group had only residual shoulders at 525nm. The presence of mature Bchl-a was indicated by the peaks at 380 and 595nm. The broadening of the 800nm peak suggests the presence of a lot of 'free' Bchl-a. The shift in the 872 peak to 880nm in UV32 suggests that

the LHII complex is absent although the shift is smaller than that observed in the typical grey mutants where the peak was shifted to 884nm. In addition the 800nm peak is not reduced greatly relative to the 880nm peak, as in other grey mutants. These observations suggest that some alterations have occurred in the environment of the Bchl-a in the membrane. This could be correlated with alterations in the carotenoid content since this is very different from the wild-type, or alternatively it could be due to altered protein-Bchl-a interactions. The semi-denaturing PAGE analysis showed that UV32 had a greatly altered pattern of pigment-protein complexes. The normal LHI/RC band and its associated aggregates were missing at the 150kD position, and two new bands occurred in the 90-150kD region. These could represent aberrant LHI/RC complex aggregates and the need to be identified with certainty using immunolabelling. UV32 also had a band running at 90kD, the position of the LHII complex. It is not possible to state whether this actually is the LHII complex or another aberrant LHI/RC aggregate which just happens to migrate to the 90kD position. As mentioned above, the spectral analysis suggested that the LHII complex was missing.

The fully denaturing PAGE analysis suggested that all the polypeptides of the normal photosynthetic apparatus were present in the membrane of UV32. Thus, it is possible that both complexes are being assembled but in an altered or less stable configuration, leading to the change observed in the semi-denaturing gel.

In NTG29 the 870nm peak was not only shifted towards 884nm, but replaced by two much smaller peaks. Again, this indicates considerable changes in the Bchl-a in situ environment, and suggests absence of LHII and changes in the LHI complex. The semi-denaturing PAGE confirmed this: the 90kD

LHII band was absent and the LHI/RC band had been replaced by aggregates (a and c) in the 90-150kD region. Again the appearance of a large amount of protein in aggregate (a) can be correlated with absence of the normal LHI/RC at 150kD. The other band (c) was unique to this mutant.

The full denaturing gel suggested that all of the polypeptides of the normal photosynthetic apparatus were present. NTG29 had a much altered carotenoid spectrum and again this correlates with a disrupted organization of the pigment-protein complexes. Thus, although all the polypeptides appear to be present in the membrane, the normal pigment-protein complexes are not formed, LHII is not present and a much altered LHI/RC is present. Further evidence that the pigment-protein complexes were altered in these two mutants came from the fact that both of them exhibited considerably enhanced autofluorescence.

In the interpretation of in vivo spectra it is important to remember that small changes in the composition and interaction of the components of the photosynthetic apparatus can reflect great alterations in the spectra observed and thus only tentative conclusions about the organization of the photosynthetic apparatus can be made from such spectra.

In addition, Sistrom and Clayton (1964) in studying a blue/green strain of Rb. sphaeroides PM8-bg58 found that when chromatophore fractions of the mutant were exposed to 1% Triton X-100, a considerable part of the Bchl was converted to a form absorbing maximally at about 870nm. This suggests that the pigment-protein complex of blue/green mutants are less stable than those with a full complement of carotenoids and are more susceptible to attack by detergents. Interpretation of the semi-

denaturing gels used here should take this into account. The altered patterns observed in mutants UV32 and NTG29 could be due to the instability of the carotenoid-depleted pigment-protein complexes in the presence of Triton X-100 and if so then the gels could not be used to make conclusions about the in vivo status of the photosynthetic apparatus.

6.6.3 Green and yellow mutants (Groups 3 and 4)

The spectra of both green and yellow mutants indicated that mature Bchl-a was not present since there were no peaks at 380/595nm. Neither did they have any peaks in the IR region of the spectrum characteristic of the presence of Bchl-a pigment-protein complexes.

The green mutants usually had a peak at 667nm presumably due to a Bchl precursor and the yellow mutants had no peak in the near-IR region of the spectrum. Members of both groups usually had a shoulder at 445/460nm indicative of the presence of residual amounts of carotenoids, but it is not possible to identify them from spectra. In addition, most of these mutants had a peak at 415/420nm which is possibly due to the presence of a flavoprotein species. Kelly (1985) isolated an unknown yellow pigment from the ICM of Rm. vanniellii. demonstrated that it was not a cytochrome and suggested that it was a flavoprotein. This species had an absorption maxima at 418nm and this peak would normally be masked by the presence of the peak due to mature Bchl-a. Similar Bchl-less mutants have been isolated from other organisms and they usually fall into two groups as observed here. Griffiths (1962) isolated more than 20 mutants showing altered chlorophyll synthesis. They fell into two main groups having pigments with long wavelength absorption maxima at about 625 and 660nm

respectively, in organic solvents. When grown on plates all mutant colonies were some shade of green. The '625nm' group were generally yellower than mutants of the '660nm' group. In addition the '660nm' mutants had a short wavelength peak at 410nm and the '625nm' mutants had a peak at 412nm. This is similar to the absorption peaks found in the 415nm region of the spectra of yellow/green mutants isolated here. The '625nm' and '660nm' mutants both had residual carotenoid peaks in the 500nm region and a similar residual pattern of absorption in the carotenoid region was observed here. Griffiths suggested that the '660nm' mutants of Rb. sphaeroides were accumulating phaeophytin-a which absorbs maximally at 667nm in ether and that the '625nm' mutants were accumulating a precursor closely related to protochlorophyll.

Oelze and Drews (1970) described a mutant of Rb. rubrum which did not form Bchl and excreted the precursors pheophorbide-a (p 667) and 2-devinyl-2-hydroxyethyl-pheophorbide-a into the medium.

Drews et al (1971) isolated another green strain Ala-pho⁻ of Rb. capsulatus which secreted a green pigment into the medium. The pigments were identified as protochlorophyll and protopheophytin and gave in situ peaks at 418, 442 and 632nm. Yen and Marrs (1976) used some Crt⁻/Bchl⁻ double mutants in the mapping of genes for carotenoid and Bchl biosynthesis in Rb. capsulatus. There were two groups designated P630-Crt⁻ which were yellow in colour, and P670-Crt⁻ which were green.

Thus the two groups of Bchl-less mutants isolated here are very similar to those isolated in Rb. capsulatus and Rb. sphaeroides. The green mutants produce and secrete precursor(s) from the later steps in the biosynthetic pathway since molecules containing the tetrapyrrole ring

absorb in the 660nm region, and the yellow mutants are presumably blocked in very early steps since no peaks are observed in the near IR region.

The semi-denaturing PAGE analysis of the green/yellow mutants also suggested the absence of the normal LHI/RC and LHII pigment-protein complex at 150 and 90kD respectively. However, the fully denaturing gels suggested that some of the polypeptides, ie the 11 and 13kD polypeptides from LHII and the 14kD polypeptide from LHII were still present in the ICMs of these mutants. In the four mutants analyzed, all were missing the three RC polypeptides and the RC-associated cytochrome (C-553) and the LHI 12kD polypeptide. This suggests that the expression of these four polypeptides may be co-ordinated.

Rb. sphaeroides mutants unable to synthesize Bchl lack the characteristic intracellular membranes associated with pigmented cells, suggesting that the mature Bchl-molecule is required for assembly of the membranes. Consequently, it would be interesting to look at the status of the ICMs of the Rm. vannielii mutants using EM techniques. Dierstein (1983) suggested that Bchl is needed to stabilize the polypeptides of the pigment-protein complexes in Rb. capsulatus.

Inhibition of Bchl synthesis in wild-type strains of Rb. capsulatus did not impair formation of mRNA for pigment-binding polypeptides, but inhibited the synthesis of these proteins as well as stable incorporation into the membrane (Klug et al. 1986). These results suggested that Bchl is necessary for the stabilization of pigment-binding proteins in the membranes and that intermediates of Bchl-synthesis affected the synthesis of these proteins.

In Rb. capsulatus and Rb. sphaeroides in the stable assembly of reaction centre and antenna pigment-protein complexes in the photosynthetic membrane depends on the synthesis of all protein and pigment components (Drews et al. 1976; Kaufman et al. 1984; Zsebo and Hearst, 1984). In Rb. capsulatus synthesis and assembly are co-ordinated (Klug et al., 1986) and the synthesis of pigment-binding polypeptides is regulated at the level of transcription (Clarke et al. 1984; Belasco et al. 1985; Klug et al. 1985). These polypeptides were found to be synthesized in a heterologous cell-free translation system (Dierstein, 1984) as well as in mutant strains defective in Bchl synthesis (Dierstein, 1983).

Thus the synthesis of these polypeptides is not dependent on Bchl, and Bchl-less mutants are able to synthesize pigment-binding protein. However, in these mutants only small amounts of pigment-binding polypeptides are synthesized although the level of specific mRNA is nearly the same as the wild-type under steady-state growth. This, combined with studies with inhibitors, suggests that precursors of Bchl synthesis influence the translation rates of pigment-binding proteins. A further effect of Bchl is the stabilization of polypeptides after assembly of complexes. Proteins of the photosynthetic apparatus, which are incorporated into the membrane fraction but not assembled into a complex because Bchl or another polypeptide of the complex are lacking, disappear from the membrane (Dierstein, 1983; Dierstein et al. 1984).

Alternatively, a block in Bchl synthesis leading to the accumulation of precursors could affect the translation of these polypeptides from mRNA as found in Rb. capsulatus (Dierstein, 1984). In Bchl-less mutants only small amounts of pigment-binding polypeptides are synthesized, although

the level of specific mRNA is nearly the same as in the wild-type under steady state growth. Addition of inhibitors of Bchl synthesis does not impair the transcription of mRNA for Bchl-binding proteins, but inhibits the synthesis of RC and B870 proteins completely and only impaired the synthesis of B800-850 proteins.

The genes for the RC and antenna structures have been cloned and sequenced in both *Rb. capsulatus* and *Rb. sphaeroides* organisms. The genes are organized in three operons: the *puf* operon codes for the reaction centre L and M polypeptides and the two LHI polypeptides and the LHII polypeptides are located on a separate *puc* operon (Kiley and Kaplan, 1987, 1983). The third RC polypeptide H is encoded by *puh* A (Donohue *et al.*, 1986a).

Kelly (1985) showed that the regulation of light-harvesting complexes by light under anaerobiosis was similar to that observed in other organisms, ie the level of LHI/RC in the ICM remained constant with variation in light intensity whereas the level of the LHII complex varied inversely with light intensity suggesting that they were controlled by different regulatory mechanisms.

6.7 Conclusions

The pigment mutants of *Rm. vannielii* fall into four main groups according to their absorption spectra. Orange/brown mutants were altered only in their carotenoid content and had apparently normal pigment-protein complexes with a full complement of polypeptides. Grey mutants were very similar to the classical blue/green mutants of the Rhodospirillaceae. Absence of carotenoids was associated with absence of

the LHII spectral form of Bchl-a. The protein patterns of the two mutants analyzed here were atypical of the group. They had a full complement of polypeptides, but contained aberrant pigment-protein complexes. These two groups of mutants suggest that the carotenoid pigments play a role in stabilizing the pigment-protein complexes since increased alteration in carotenoid content is associated with increased instability of complexes. The green/yellow mutants lacked mature Bchl-a. Green mutants are thought to produce a Bchl-precursor containing a tetrapyrrole ring and yellow mutants are blocked earlier in the Bchl biosynthetic pathway. These mutants suggest that Bchl-a is required for the assembly of pigment-protein complexes. In its absence, some of the LH polypeptides are still incorporated into the membrane.

Since the main thrust of this project was the development of a system for genetic analysis, the characterization of the pigment mutants is incomplete. Further work should include: the identification of the intermediates of carotenoid and Bchl synthesis being accumulated using TLC and HPLC techniques; EM analysis to assess any changes occurring in the lamellate membrane system of the mutants, and the positive identification of the RC and LH polypeptides using immunolabelling techniques with antibodies raised against analogous polypeptides in Rb. capsulatus or Rb. sphaeroides.

This work demonstrates that a wide variety of mutants with lesions affecting the photosynthetic apparatus can be isolated. Specific mutants tailored for research into the photosynthetic apparatus of Rm. vannielii and its role in the differentiation cycle should be isolated as desired in future. The next chapter considers an alternative approach for the generation of mutants - transposon mutagenesis.

CHAPTER 7

TRANSPOSON MUTAGENESIS

7.1 Introduction

Transposons are mobile genetic elements which can move to new locations in DNA molecules, independently of homologous recombination. Many such elements have been found in prokaryotes and eukaryotes. They carry genes necessary for transposition, and often carry selectable markers, eg resistance to antibiotics or heavy metals, sugar-utilization or pathogenicity determinants. A number of transposons isolated from resistance plasmids of E. coli and Pseudomonas spp. have been described (Kleckner et al. 1977; Berg D and Berg C, 1983; Mills, 1985). The use of transposons offers certain advantages over traditional methods of genetic manipulation. The most common use of transposons is to generate stable mutations by insertional inactivation of a gene, thereby causing complete loss of gene function, and simultaneously allowing 'tagging' of the gene of interest with a selectable marker. Other examples of their use include the marking of non-selectable genes (Kleckner et al. 1977); the generation of deletions (Berg D and Berg C, 1983); the cloning of non-selectable genes (Purucker et al. 1982; Niepold et al. 1985); the study of operon organization (Berg C et al. 1979; de Bruijn and Lupski, 1984) and use as a portable region of homology for directed chromosome transfer (Banfalvi et al. 1983; Pischl and Farrand, 1983).

A wide range of transposons have been isolated and characterized but the properties of Tn5 have been studied most extensively (Berg D and Berg C, 1983; de Bruijn and Lupski, 1984), and it is the element most commonly used for genetic manipulation in Gram-negative bacteria. The advantages of Tn5 include a low specificity of insertion in most organisms (Shaw and Berg, 1979; Berg D et al. 1983), availability of its complete nucleotide sequence, well characterized gene products (Rothstein et al.

1981; Rossetti et al. 1984), the production of relatively stable insertions (Berg D and Berg C, 1983; Berg C et al. 1983), and the generation of completely polar mutations (Berg C et al. 1979; Berg D et al. 1980; de Bruijn and Lupski, 1984). Mutant isolation is relatively easy since Tn5 transposes at a high frequency, (10^{-2} to 10^{-3} per cell in *E. coli*). However, Tn5 does not transpose efficiently in all Gram-negative species, eg *Rhizobium meliloti* (Meade et al. 1982; Forrai et al. 1983). The transposition efficiency of Tn5 is strain-dependent in some species (Forrai et al. 1983). Insertional specificity of Tn5 has been reported in *Acinetobacter calcoaceticus*, where it was shown to have a single site of insertion in the chromosome (Singer and Finnerty, 1984). In addition to Kanamycin resistance, Tn5 also expresses Bleomycin resistance (Genilloud et al. 1984; Collis and Hall, 1985) as well as Streptomycin resistance in many non-enteric species (O'Neill et al. 1984).

Transposon mutagenesis

To achieve transposon mutagenesis a 'suicide vector' (van Vliet et al. 1978) is required. Upon transfer to the recipient cell, such vectors fail to replicate, and selection of the markers coded by the transposon dictates that all survivors (except spontaneous mutants) carry transposon insertions. Examples of suicide vectors include: plasmids of narrow host-range (Simon et al. 1983a, b; Ely, 1985; Whitta et al. 1985; Boulnois et al. 1985); Phage P1 (Kuner and Kaiser, 1981; Quinto et al. 1984) and λ phage derivatives (Palva and Liljestrom, 1981; de Vries et al. 1984). Three narrow host-range vectors were studied in the present work, namely pJB4JI (Berlinger et al. 1978), pSUP2021 (Simon et al. 1983a) and pLG221 (Boulnois et al. 1985). In addition, an attempt was

made to transfer the receptor/lamB gene on plasmid pTROY413 to Rm. vannielii with the aim of exploiting a λ -based, transposon mutagenesis system (de Vries et al. 1984; Ludwig, 1987).

pJB4JI - a Mu-carrying suicide plasmid

Suicide vectors based on broad host-range plasmids carrying the Mu prophage have been used in the mutagenesis of a variety of bacterial species representing groups ranging from the enterics (Chatterjee et al. 1983) through pseudomonads (Boucher et al. 1981; Stapleton et al. 1984), rhizobia and related species (Beringer et al. 1978; Pischl and Farrand, 1983) to the Rhodospirillaceae (Weaver and Tabita, 1983). The use of such suicide vectors in Caulobacter crescentus (Ely and Croft, 1982; Bellofatto et al. 1984) and Rhodobacter sphaeroides (Weaver and Tabita, 1983) is perhaps of most relevance to the present work.

Reasons for the instability of Mu-carrying plasmids in species other than E. coli are discussed by van Vliet et al. (1978) and Casey et al. (1983). However, the destabilizing effect of Mu is poorly understood.

The most commonly used Mu-carrying suicide plasmid is pJB4JI (Beringer et al. 1978). This R751-based replicon carries the Mu ϕ^+ prophage with a Tn5 element inserted in the tail-protein genes of Mu (Hirsch and Beringer, 1984). Thus, E. coli strains containing pJB4JI are Mu-immune yet unable to produce viable Mu phage (Beringer et al. 1978). Upon transfer to many Gram-negative species, pJB4JI is unstable, and transposition of Tn5 to the chromosome may be selected. However, in some cases, this transposition event may be accompanied by insertion of Mu DNA, thus complicating genetic analysis and subsequent directed cloning

experiments (Meade *et al.* 1982). Further complications are that pJB4JI is maintained in some species, including *Rb. capsulatus* (Kaufmann *et al.* 1984) and *Rb. sphaeroides* (Weaver and Tabita, 1983). In the latter case the plasmid was lost using a serial passage on non-selective media. The genetical complications arising in the use of Mu-based suicide vectors are discussed by Casey *et al.* (1983) and O'Neill *et al.* (1983). Despite these problems pJB4JI has proved useful in a wide range of species.

Transposon delivery vectors based on narrow host-range plasmids

An alternative strategy for transposon mutagenesis is to use plasmids that have a narrow host-range (Simon *et al.* 1983a; Boulnois *et al.* 1985; Ely, 1985; Whitta *et al.* 1985). Simon *et al.* (1983a, b) constructed derivatives of cloning vectors pBR325 and pACYC184 which carried the *oriT* region ('mob') from RP4, permitting mobilization by RP4 functions in *trans* (Clarke and Warren, 1979). pSUP2021 is a *mob*⁺ derivative of pBR325 with Tn₅ inserted in its tetracycline resistance gene. pSUP2021 is a suicide vector due to the restricted host range of the ColE1 prototype plasmid which does not replicate outside *E. coli* and closely related species, (O'Neill *et al.* 1983; Ely, 1985). pSUP2021 or similar vectors have been successfully used in a range of organisms including various *Rhizobium* spp. (Simon *et al.* 1983a); *Pseudomonas* spp. (Lam *et al.* 1985) and members of the Rhodospirillaceae (Kaufmann *et al.* 1984; Wright *et al.* 1987; Klipp *et al.* 1988).

Boulnois *et al.* (1985) constructed a narrow host range vector pLG221 based on the prototype IncI plasmid ColIb. The normal hosts for this plasmid are *E. coli* and closely-related species. pLG221 is a Tn₅-carrying derivative of ColIb *drd-1*, a mutant ColIb plasmid that

constitutively expresses conjugal ability. pLG221 has been used for transposon mutagenesis in a variety of Pseudomonas spp, Agrobacterium tumefaciens and Methylophilus methylotrophus (Boulnois et al., 1985). This plasmid has the advantage over pSUP2021-type plasmids of being self-transmissible.

Bacteriophages as suicide vectors

Transposon mutagenesis has been carried out using phages as delivery vehicles in various bacterial species (Kuner and Kaiser, 1981; Palva and Liljestron, 1981; Belas et al. 1982; Berg D and Berg C, 1983; Harkki et al., 1984; de Vries et al. 1984). The most important examples are the use of P1 and these are briefly described below.

P1 The host range of P1 is normally limited to E. coli and closely-related enterics. Nonetheless, it is possible to select P1-sensitive mutants of different bacterial species using P1-derivatives carrying antibiotic-resistance genes (Goldberg et al. 1974; Murooka and Harada, 1979; Quinto et al. 1984). Such mutants permit the efficient absorption of P1 phage particles and the injection of the DNA carried by them, but rarely provide the host functions necessary for replication. P1 has been used to achieve transposon mutagenesis of various enteric bacteria as well as the non-enterics Myxococcus xanthus (Kuner and Kaiser, 1981) and Vibrio harveyi (Belas et al., 1982).

Lambda Generally, the host range of Lambda does not extend beyond E. coli K12, and methods for the selection of sensitive mutants of other strains or species are not available. A single receptor protein, the lamB gene product facilitates absorption and is sited in the outer membrane (Silhavy et al. 1983). The lamB gene has been cloned into

multicopy vectors and introduced into a number of bacterial species (Clement *et al.* 1982; Harkki and Palva, 1985; de Vries *et al.* 1984). This approach led initially to the construction of sensitive derivatives of bacterial species such as Salmonella typhimurium and Klebsiella pneumoniae. This was achieved using plasmids pTROY9 and pTROY11 carrying the native lamB gene (de Vries *et al.* 1984). Later the range was extended to Agrobacterium, Pseudomonas and Rhizobia spp. using pTROY413 which carried manipulated lamB genes coding for receptors that functioned normally in these species (Ludwig, 1987).

In the absence of replication, the ability of λ to inject its DNA can be exploited for transposon mutagenesis. Deletion derivatives of λ , which cannot lysogenise or replicate are commonly used for the introduction of transposons to chromosomal or plasmid-borne genes of E. coli (Kleckner *et al.* 1977; Berg D and Berg C, 1983; de Bruijn and Lupski, 1984).

The advantage of suicide vectors over plasmid vectors for transposon mutagenesis are as follows. Firstly, many transposon-carrying derivatives of λ are available allowing the use of any transposon of choice (Kleckner *et al.* 1977; Berg D and Berg C, 1983; de Bruijn and Lupski, 1984; Way *et al.* 1984), including transposons developed as promoter-probes (Way *et al.* 1984). Secondly, phage infection takes place more rapidly than plasmid transfer and allows the formation of 'random' transposon-induced mutants without concomitant sibling formation.

Since the various transposon delivery systems discussed here show great variability in efficiency from species to species, and there is no assurance that any one of them would work in Rm. vannielii, it was decided to try a range of vectors. This chapter describes attempts to

carry out transposon mutagenesis using pJB4JI, pSUP2021, pLG221 and to make Rm. vanniellii λ -sensitive using pTROY413.

7.2 Selection of transconjugants on plates using the 'gas-bag' incubation technique

Potts (1980) demonstrated the transfer of R68.45 to a 'P+S' strain of Rm. vanniellii with the expression of Ampicillin (Ap) but not Tetracycline (Tc) or Kanamycin (Km) resistance determinants of the plasmid.

The literature records several procedures for bacterial conjugation. These include liquid mating (Miller and Kaplan, 1978), mating on solid medium (Sistrom, 1977), patch-mating on solid medium (Tucker and Pemberton, 1979b) and filtration onto a filter followed by incubation on solid media (Ely, 1979). Potts (1980) compared the efficiencies of liquid and solid media matings and also aerobic and anaerobic mating, concluding that aerobic mating carried out on solid medium was the most efficient. In the present study, the effects of varying conditions in conjugation on solid media were studied. The effects of aerobic/dark conditions were compared with anaerobic/light and anaerobic/dark conditions. The methods of Sistrom (1977) and Ely (1979) were also tried. In all cases the post-conjugation selection plates were incubated using the 'gas-bag' technique and no colonies exhibiting antibiotic-resistance were found.

7.3 Transposon mutagenesis using E.coli HE102 (pJB4JI)

7.3.1 Conjugation of E. coli (HE102) (pJB4JI) and Rm. vanniellii with selection of transconjugants in tubes

Following the success in isolation of spontaneous antibiotic resistant mutants using the agar-tube technique (Section 4.2.2), it was decided to select transconjugants in tubes. A standard method of mating was adopted and is described in Section 2.16. Suspensions of post-conjugation cells were used to inoculate tubes of molten medium as described in Section 4.2.2. In this case, the media contained antibiotics for selection and counter-selection. Table 7.3.1 shows the analysis of colonies obtained after three conjugations using E. coli HE102 (pJB4JI) as a donor to three different strains of Rm. vanniellii. There was a marked difference between experiments 1 and 2; each of the frequencies in experiment 1 was about 10-fold greater than the corresponding one in experiment 2. A most interesting observation was that the frequency of Km or Streptomycin (Sm) resistant colonies was higher than frequencies for spontaneous resistance to these antibiotics determined in Chapter 4, or those observed in controls in experiment 3 (Table 7.3a). The spontaneous antibiotic resistance frequency was found to be generally about 4×10^{-10} , except for Spectinomycin (Sp) which had previously been calculated as 5.8×10^{-10} but here was found to be about 4×10^{-7} . The frequencies of resistance to Sp were much higher than those to other antibiotics and the control in experiment 3 suggested that these were due to spontaneous mutation.

In addition, the selection of colonies exhibiting simultaneous resistance to both Km and Sm in experiments 1 and 2 (Table 7.3.1)

Table 7.3.1 Transposon mutagenesis of *Rm.vannielii* using pJB4JI with selection of transconjugants in tubes.

Donor <u>Rm.</u> <u>vannielii</u>	Recipient <u>E.coli</u>	Counter- selection (Antibiotic $\mu\text{g ml}^{-1}$)	Selection	Frequency of transcon- jugants per recipient	
				Experiment 1	Experiment 2
RB1 (Rm5, Sm ^r)	J53 <u>nal</u> (pJB4JI)	Sm 100	Km 20	1.32×10^{-8}	1.28×10^{-9}
		Sm 100	Sp 100	3.95×10^{-6}	1.02×10^{-7}
		Sm 100	Km 20,	$<2.5 \times 10^{-10}$	$<2 \times 10^{-11}$
Sp 100					
RB2 (Rm5, Nal ^r)	J53 <u>nal</u> (pJB4JI)	Nal 100	Km 20	1.57×10^{-8}	4.46×10^{-9}
		Nal 100	Sp 100	6.44×10^{-6}	1.34×10^{-7}
		Nal 100	Sm 30	5.8×10^{-7}	3.35×10^{-8}
		Nal 100	Km 20, Sm 30	1.28×10^{-9}	6.09×10^{-9}
		Nal 100	Km 20, Sp 100	$<1.5 \times 10^{-10}$	$<1.5 \times 10^{-11}$
		Nal 100	Sp 100, Sm 30	$<1.5 \times 10^{-10}$	$<1.5 \times 10^{-11}$
		Nal 100	Sp 100, Sm 30, Km 20	$<1.5 \times 10^{-10}$	$<1.5 \times 10^{-11}$
Experiment 3					
RB3 (P+S, Sm ^r)	J53 <u>nal</u> (pJB4JI)	Sm 100	Km 20	1.35×10^{-8}	
		Sm 100	Sp 50	5×10^{-6}	
		Sm 100	Km 20, Sp 50	1.66×10^{-9}	
RB3	none (control)	Sm 100	Km 25	$<4 \times 10^{-10}$	
		Sm 100	Sp 50	4×10^{-7}	
		Sm 100	Km 20, Sp 50	$<4 \times 10^{-10}$	

Conjugation conditions: PYM agar, O/N, 30°C. Transconjugants selected in agar columns.

suggested the presence of Tn5 since the transposon confers resistance to both of these antibiotics in non-enteric bacteria. It was unlikely that double mutations giving resistance to two antibiotics could be selected at the frequency observed here. Alternatively these doubly-resistant colonies could be due to a single mutation conferring resistance to aminoglycoside antibiotics in general. In experiment 3 (Table 7.3.1) the frequency of resistance to Km was two orders of magnitude greater than that of the control where the frequency of spontaneous Sp resistance frequency was relatively high at 4×10^{-7} . Again the frequency of simultaneous double resistance to Sp and Km was too high to be attributable to spontaneous mutation.

7.3.2 Screening of colonies from tubes after pJB4JI conjugation

Table 7.3.2 shows the results of screening a selection of the colonies from experiments 1 and 2 (Table 7.3.1) for selected and non-selected markers. To avoid or decrease the problems encountered in using the 'gas-bag' technique colonies were stabbed into the agar a few times for each colony. To avoid condensation gathered in the petri dish lids coming into contact with the agar surface, large 18cm plates were used. These were deeper than the usual 9cm plates and a large number of colonies could be stabbed into each plate.

In both experiments there were significant replating losses and the reason for this is not understood but it may be due to the use of the 'gas-bag' incubation technique. However, using RB2 as the recipient, about 40% of the Km-resistant colonies were also resistant to Sm. This was significantly higher than values obtained for any other co-resistance to a non-selected marker, all of which were at 15% or less.

Table 7.3.2 Screening of colonies from tubes after transposon mutagenesis with pJB4JI

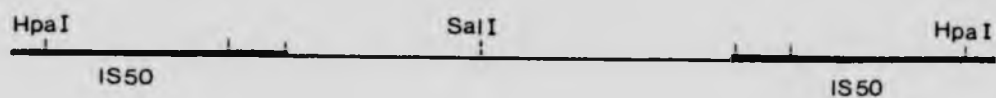
Selection (anti- biotic $\mu\text{g ml}^{-1}$)	Experiment (1/2, Table 7.3.1)	Recipient (<u>Rm.</u> <u>vannielii</u>)	Screening of selected and non- selected markers (antibiotic $\mu\text{g ml}^{-1}$)		
			selected	non-selected	
Km 20	1	RB1	Km 20	Sp 100	Sm 30
		RB2	100 %	0	R
	2	RB1	100 %	0	38 %
		RB2	81 %	0	R
		RB2	100 %	0	41 %
		RB2			
Sp 100	1	RB1	Sp 100	Km 20	Sm 30
			100 %	38 %	R
	2		92 %	15.4 %	0
		RB1	81 %	0	R
			92 %	7 %	0
Sm 30	1	RB2	Sm 30	Km 20	Sp 100
			92 %	7.7 %	0
	2	RB2	92 %	15.4 %	7.7 %
Km 20, Sm 30	1	RB2	Km 20	Sp 100	
			100 %	0	
	2		Sm 30		
			100 %		
		RB2	Km 20	Sp 100	
			100 %		
			Sm 30	8.3 %	
			75 %		

Agar columns were dissected and colonies stabbed into PYM agar, containing the selective antibiotics, in 18 cm petri dishes. Phototrophic incubation conditions were used. R - intrinsic resistance to Sm by Rm.vannielii RB1.
% - % No. of colonies that regrow/No. of colonies screened.

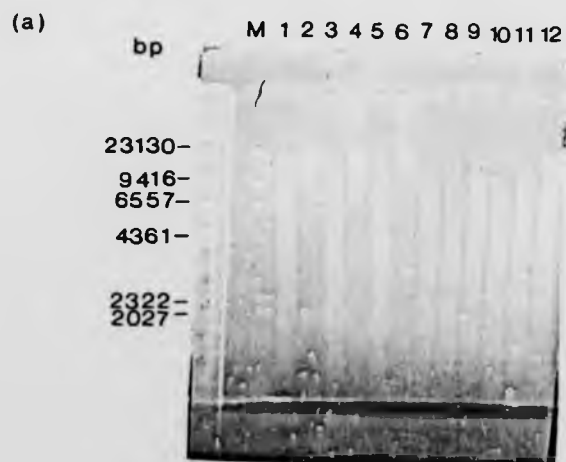
All colonies exhibiting double resistances were screened for the plasmid-coded marker Gentamycin (Gm) ($5\mu\text{g ml}^{-1}$), and only one was found to be resistant. Given the high frequency of co-resistance to Sm on screening Km-resistant colonies, it is surprising that the frequency of co-resistance to Km on screening Sm-resistant colonies was not higher. These results suggested that transposon insertion was occurring at about 2.8×10^{-8} per recipient with a lower background frequency of plasmid integration or maintenance. However, no mutants were observed amongst the Kanamycin-resistant colonies, but this may be a reflection of the growth conditions which would select against photosynthetically-incompetent mutants.

7.3.3 Demonstration of the physical presence of Tn5

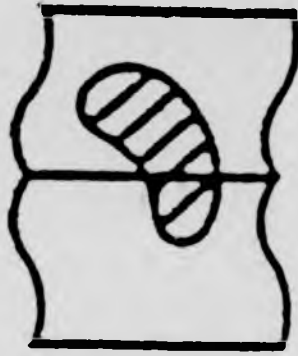
Southern blot analysis (Figure 7.3.3)(with a ^{32}P labelled HpaI internal fragment from Tn5) of restricted total genomic DNA from a selection of the antibiotic-resistant strains and the parental strain, verified the presence of Tn5 in the transconjugants. The transposon was present in four out of five Km/Sm-resistant strains, and was absent in the Km/Sm/Gm-resistant strain and also absent in the parent strain. In those strains that carried Tn5, the Southern blot analysis showed that the transposon was inserted in different fragments of the genome. The multiple bands observed in tracks 10 and 12 when digesting with SalI are most likely due to partial digestion of the DNA. Alternatively they may be due to secondary insertion sites, of Tn5 or its 1S50 inverted repeat sequences, elsewhere on the chromosome. The latter seems unlikely since digestion with ClaI gave a single band as expected.



Cleavage map of Tn5. The transposon comprises 5.7 Kb of DNA. Digestion of chromosomal DNA carrying a single Tn5 insert with SalI should give two fragments containing Tn5 sequences whereas digestion with ClaI will yield only one such fragment.



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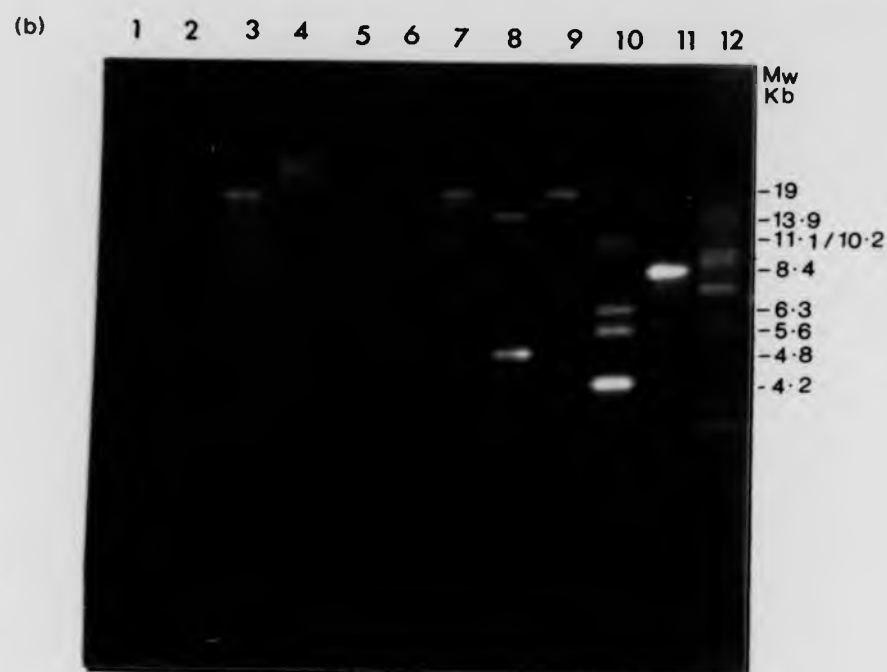


Figure 7.3.3b Demonstration of the physical presence of *Tn5* in *Rm.vannielii* chromosomal DNA

Total genomic DNA from a selection of Km and Sm resistant transconjugants and the parental strain were digested with either *Cla*I or *Sal*I and the fragments separated on a 0.6% agarose gel (a). Southern blot analysis using a ³²P-labelled *Hpa*I internal fragment from *Tn5* gene gave this autoradiograph (b).

Source of DNA	Restriction enzyme:	
	<i>Cla</i> I	<i>Sal</i> I
Tracks		
Wild type RB2	1	2
Sm ^r Km ^r	3	4
Sm ^r Km ^r Gm ^r	5	6
Sm ^r Km ^r	7	8
Sm ^r Km ^r	9	10
Sm ^r Km ^r	11	12

<photo.-contact print>

7.3.4 Conjugation of *E. coli* HE102 (pJB4JI) and *Rm. vannielii*
RB4 with selection of transconjugants on plates using
microaerophilic growth conditions

In a conjugation experiment to transfer pJB4JI to RB4, the mating was performed as described in Section 2.16, and selective plates were incubated under microaerophilic (1% O₂) conditions. Colonies resistant to Sm at 25 µg ml⁻¹ were isolated at a frequency of 3.7 x 10⁻⁶ per recipient, and one colony out of 99 had altered pigment content (Figure 7.3.4a). Screening revealed that 76% of the colonies were also resistant to Km at 25 µg ml⁻¹ and showed sensitivity to Sp or Gm. None of those colonies that were resistant to Km were resistant to Sm at a higher concentration (50 µg ml⁻¹). These were putative transposons. Approximately 18% of the colonies were resistant to Sm at high concentration yet were sensitive to Km, Sp and Gm. These were probably spontaneous Sm-resistant mutants. The remaining 6% were resistant to Km, Sp, Gm and Sm at low levels, suggesting that they were maintaining the plasmid. Plates showing this heterogeneity of resistance are shown in Figure 7.3.4b.

7.3.5 Discussion of the use of pJB4JI

Upon transfer of pJB4JI to *Rm. vannielii* a high percentage of transconjugants appeared to maintain the suicide vector either as an independent replicon or integrated in the chromosome. The physical status of maintained plasmids was not investigated. The problems that have been encountered in using pJB4JI as a suicide vector in other organisms, include plasmid maintenance (Casey *et al.* 1983) and the induction of genetic changes secondary to the transposition of Tn5



Figure 7.3.4a Colony with altered pigmentation amongst Sm^r trans-conjugants after transfer of pJB4JI to Rm.vannielii RB4

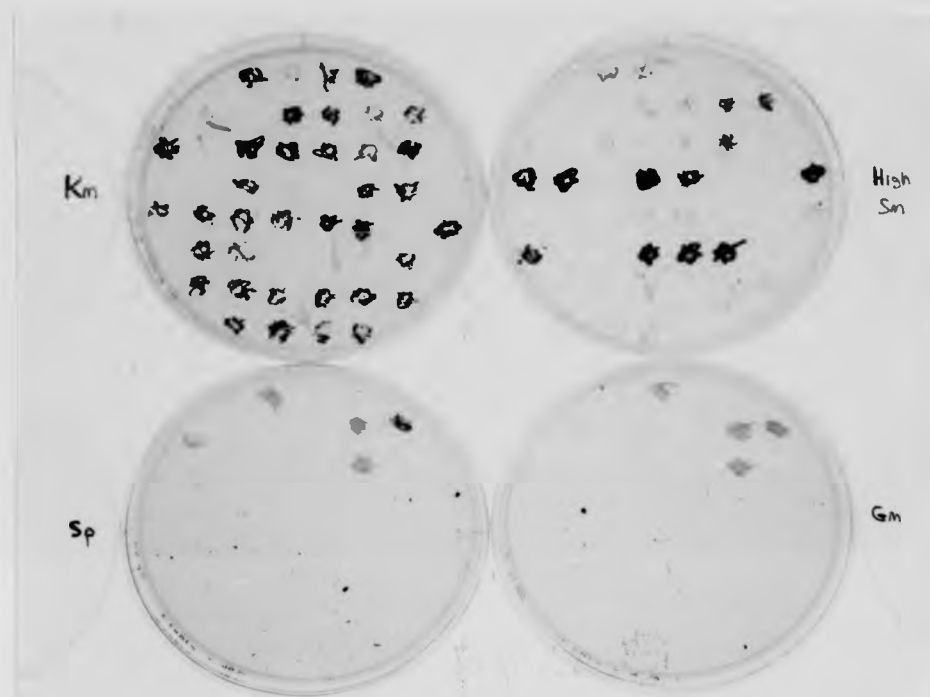


Figure 7.3.4b Transconjugants obtained after transfer of pJB4JI into Rm.vannielii RB4 exhibit heterogenous antibiotic resistance patterns

Transconjugants selected on Sm $25 \mu\text{g ml}^{-1}$ were screened on Km $25 \mu\text{g ml}^{-1}$ (1) Sm $50 \mu\text{g ml}^{-1}$ (2), Sp $20 \mu\text{g ml}^{-1}$ (3) and Gm $5 \mu\text{g ml}^{-1}$ (4). Plates were incubated under microaerophilic $1\% \text{ O}_2$, dark conditions.

(Meade et al. 1982). The Mu-induced failure of RP4-type plasmids to replicate in many Gram-negative species can sometimes be overcome (Boucher et al. 1977) and stably-replicating mutants often cause severe problems (Simon et al. 1983a). Moreover, simultaneous transposition of Mu and Tn₅ sequences has been observed in many cases, and complicates the genetic analysis and subsequent use of the mutants (Meade et al. 1982; Forrai et al. 1983). Transposition of Mu DNA was not investigated here.

Nevertheless, this was the first demonstration of transposon mutagenesis in Rm. vannielii and despite the problems with the vector the results indicate that Tn₅ could be inserted non-specifically in the genome. In the absence of a superior delivery system, pJB4JI could be used to isolate mutants.

7.4 Transposon mutagenesis using E. coli S17-1 (pSUP2021)

7.4.1 Introduction

This transposon delivery system, developed by Simon et al. (1983a,b), consists of an E. coli donor strain carrying the transfer genes of the broad host-range IncP-type plasmid integrated into the chromosome and a vector plasmid derived from pBR325 containing the IncP-type specific recognition site for mobilization (mob site). The vector is mobilized with high frequency from the donor strain and is unable to replicate in strains outside the enteric bacterial group.

7.4.2 Selection of transconjugants in tubes

After conjugation carried out as described in Section 2.16, cells harvested from filters were suspended in agar columns containing selective antibiotics. Tubes were incubated in the illuminated waterbath described in Appendix I. Colonies appeared after 4-5 days incubation. The frequency of Km-resistant colonies after conjugation was 1.75×10^{-8} per recipient, whereas in a control the frequency of spontaneous Km-resistant colonies was $>2 \times 10^{-10}$. The agar columns from the tubes were dissected as described previously and the colonies restreaked on plates containing Km. This was prior to the development of the microaerophilic growth technique, so the 'gas-bag' technique was used to incubate the plates. No regrowth of the Km-resistant colonies occurred on plates. The stimulation of the frequency of Km-resistant colonies observed after conjugation suggested that either the plasmid or the transposon were present in Rm. vanniellii. Since pSUP2021 is a narrow host range plasmid it is very unlikely that it was being maintained as an independent replicon in Rm. vanniellii. No mutant colonies were observed in any of the tubes.

7.4.3 Selection of transconjugants on plates under microaerophilic conditions

Table 7.4.3 shows the results of an experiment in which the post-conjugation selection was carried out on plates under microaerophilic conditions. Km-resistant Rm. vanniellii colonies occurred at a frequency of 2×10^{-9} per recipient. Altering the donor-to-recipient ratio did not alter this frequency. The spontaneous mutation rate was 3×10^{-11} , so conjugation stimulated the frequency of Km-resistance 100-fold. The

Table 7.4.3 Transposon mutagenesis of Rm.vannielii using pSUP2021 with selection of transconjugants under microaerophilic conditions

Donor	Recipient	Ratio (Donor: Recipient)	Frequency (per recipient) of Km ^r colonies
<u>E.coli</u> S17-1 (pSUP2021)	<u>Rm.vannielii</u> RB4	1:1	2×10^{-9}
<u>E.coli</u> S17-1 (pSUP2021)	<u>Rm.vannielii</u> RB4	10:1	2.5×10^{-9}
none (control)	<u>Rm.vannielii</u> RB4	-	3×10^{-11}
<u>E.coli</u> S17-1	<u>E.coli</u> HE123	-	1×10^{-5}
none (control)	<u>E.coli</u> HE123	-	3.8×10^{-9}

In preliminary experiments to find conditions in which conjugation occurred, parameters that were varied included light vs. dark, aerobic vs. anaerobic; incubations and conjugation time. The technique described in section 2.16 was the first to give any transconjugants and it yielded enough colonies for screening purposes so it used subsequently. Transposon mutagenesis using this plasmid and technique was carried out several times and Km^r colonies were obtained at frequencies between 2×10^{-4} and 1.5×10^{-6} per recipient.

other controls showed that the donor was transfer-competent and transfer to another *E. coli* strain (HE123) occurred at a frequency of 1×10^{-5} relative to a spontaneous mutation frequency of 3.8×10^{-9} . There were a few pigment mutants on the plates at a frequency of 0.91%. Screening for non-selected markers showed that 50% of the Km-resistant colonies were also resistant to Chloramphenicol (Cm) at $25 \mu\text{g ml}^{-1}$ but none of them exhibited resistance to Ap at $50 \mu\text{g ml}^{-1}$. It is possible that the MIC for Ampicillin was lower than that found in *E. coli* but this was not investigated. In a screen of 100 colonies for auxotrophy on PM and PYM no mutants were isolated. Five pigment mutants were isolated and all were resistant to Km and Sm but sensitive to, the plasmid markers, Cm and Ap.

In another conjugation, Km-resistant transconjugants occurred at a frequency of 1.36×10^{-6} per recipient over a control value of 4×10^{-10} , and 0.36% were pigment mutants. Thus there was a big variation in the efficiency of transfer between different conjugation experiments.

7.4.4 Conjugations using pLG221 and pTROY413

Attempted transposon mutagenesis using pLG221 (Boulnois *et al.* 1985) resulted in Km-resistant colonies at a frequency of 1.4×10^{-6} per recipient compared to a control in which spontaneous resistant mutants occurred at a frequency of 1.2×10^{-10} . Screening of the colonies on Sm showed that none of them were resistant to this antibiotic. It is possible that the transposon had been altered in some way so that Sm resistance was no longer expressed in non-enterics. This possibility was not investigated. As the plasmid had no markers other than those carried by the transposon, plasmid maintenance/integration could not be

discriminated from transposon insertion by screening alone. No pigment mutants were observed amongst the 303 Km-resistant colonies. No auxotrophs were found in 200 colonies screened. This plasmid was not used again and the transconjugants were not analysed further.

In an attempt to make Rm. vanniellii λ -sensitive, the plasmid pTROY413 (Ludwig, 1987) was mobilized by the "helper" plasmid pRK2013 in a conjugation with Rm. vanniellii RB4. After selection on Tc-containing plates, no colonies were observed and the frequency of transfer/maintenance was calculated to be $<1.25 \times 10^{-8}$ per recipient.

7.4.5 Discussion of the use of pSUP2021

The results show that pSUP2021 can be used as a transposon delivery vector for Rm. vanniellii. Even when the frequency of transconjugants was low (2×10^{-9} per recipient), sufficient numbers of Km-resistant colonies were obtained by plating out larger volumes of the conjugation mixture. 1% of the transconjugants appeared to be pigment mutants. The observation that 50% of the transconjugants were Cm-resistant suggested that the vector was either being maintained or integrated into the chromosome. This is in contrast to the results obtained by Simon *et al* (1983a). pSUP2021 was transferred to Rhizobium meliloti at a frequency of 10^{-4} per recipient and all of the several hundred colonies tested were Cm-sensitive. Since the basic replicon pBR325 has a very narrow host-range it is likely that the Cm-resistance observed here was due to an integration event. This was not investigated further but such an integrated plasmid carrying the transfer genes of RP4 could be potentially useful in chromosome mobilization.

7.5 Analysis of transposon-induced pigment mutants

7.5.1 Spectrophotometric analysis of cell free extracts

Five pigment mutants were isolated as a result of transposon mutagenesis of *Rm. vannielii* RB4 using pSUP2021 as a suicide vector. Of these, four could be placed in the third class of mutants described in Chapter 6 and were green in colour. One of these ('Tn5I') was chosen for further study and its spectrum is compared to the wild-type spectrum in Figure 7.5.1(1). The spectrum has none of the peaks characteristic of mature Bchl-a at 380/595nm nor any in the ultraviolet region indicative of this pigment in pigment-protein complexes. The only significant peak occurs at 667nm and this is likely to be a precursor of Bchl. The fifth mutant, 'Tn5II', had a group one type spectrum (Figure 7.5.1(2)) with alterations mainly in the carotenoid region of the spectrum. The 560nm peak was totally absent. All the peaks indicative of mature Bchl-a and its presence in pigment-protein complexes were present. However, the 800nm peak was reduced relative to the 870nm peak suggesting a reduction in the amount of LHII.

7.5.2 Semi-denaturing PAGE analysis of pigment mutants

'Tn5I' and 'Tn5II'

Figure 7.5.2 shows the semi-denaturing PAGE analysis of the two mutants, 'Tn5I' and 'Tn5II' compared to the wild-type, all grown under microaerophilic conditions. This clearly shows that 'Tn5I' has no assembled pigment-protein complexes as the major bands at 150kD and 90kD, (LHI/RC and LHII respectively), were absent. In addition, there were no major non-wild-type bands in the 90-150kD region as observed with the green/yellow UV and NTG-induced mutants from group 3 as

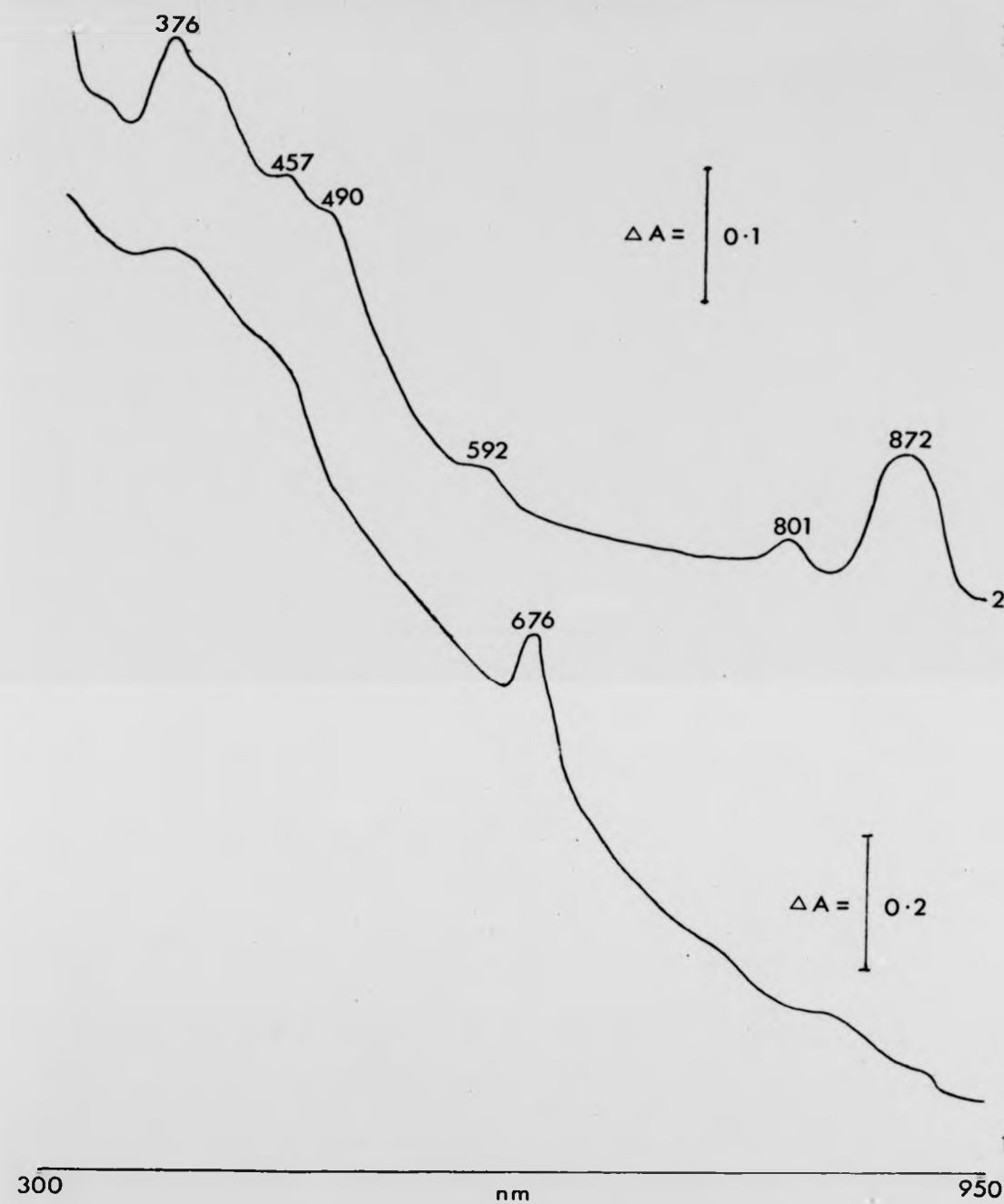


Figure 7.5.1 Absorption spectra of Tn_5 -induced pigment mutants

Room temperature absorption spectra of cell free extracts of ' Tn_5I ' (1) and ' Tn_5II ' (2). Cells were grown under microaerophilic/dark conditions with Kanamycin.

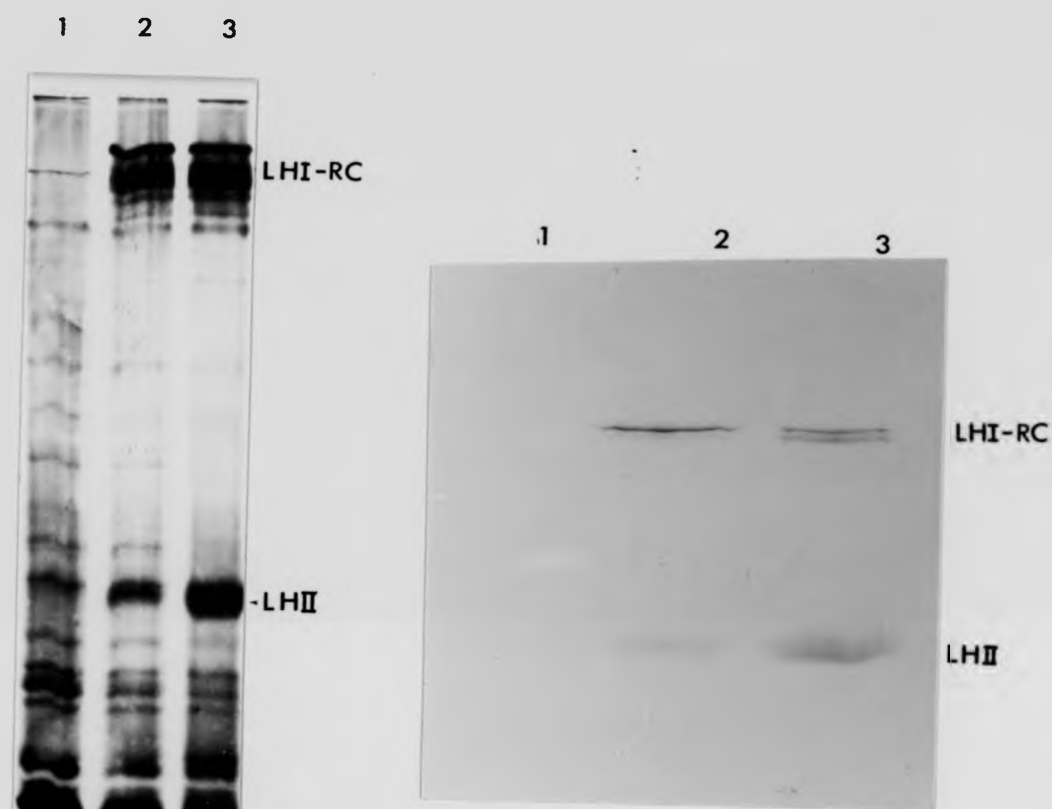


Figure 7.5.2 Intra-cytoplasmic membrane protein profiles of Tn_5 -insertion pigment mutants

PAGE with Semi-denaturing conditions was used to separate pigment-protein complexes in ICM samples prepared from microaerophilically grown cells
 Tracks: (1) ' Tn_5I '; (2) ' Tn_5II '; (3) Wild type RB4. Acrylamide concentration 7.5% (w/v). Loading - 300 μ g protein/track. Gel was photographed unstained (right) and silver stained (left).

described in Chapter 6.

The brown/orange mutant 'Tn5II' was very similar to the wild-type in respect of its protein profile. The pattern in the 150kD region was very similar suggesting that the LHI complex was not affected by the transposon insertion. However, the 90kD band was considerably reduced in size compared to the wild-type. This correlated well with the spectrum for this mutant and suggested a correlation between the reduction in carotenoid content and the LHII complex as discussed in Chapter 6.

7.6 Reversion of mutant 'Tn5I'

The spectral and PAGE analysis indicated that the mutant 'Tn5I' was photosynthetically-incompetent since none of the normal light-harvesting complexes were present in the ICM. This was verified by trying to grow the mutant under photosynthetic conditions. It failed to grow on plates incubated in the light and, in tubes, growth was restricted to the microaerophilic zone of the agar column about 2mm below the surface. Since it could only grow microaerophilically, it was possible that attempting growth in the light/anaerobically could be used as a positive selection for revertants. In an initial attempt, a lawn of mutant cells was plated out on PM without Km and incubation carried out in a gas-bag. No revertant colonies had appeared after 7 days incubation. Next, the tube method was used, mutant cells were suspended in PM agar without Km and the tubes were incubated in the perspex tank as described before. Fully pigmented revertant colonies grew in the anaerobic region of the tubes after 4/5 days incubation, whereas the mutant only grew in a narrow band in the anaerobic region of the tubes. Revertants occurred at a frequency of 4.76×10^{-8} and on screening 95 such colonies, 35% were

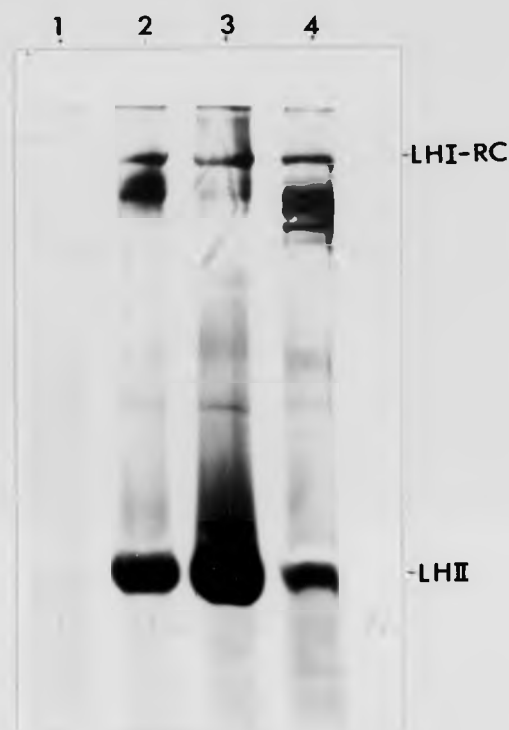


Figure 7.6 Intra-cytoplasmic membrane protein profiles of 'Tn₅I'-revertants

PAGE with semi-denaturing conditions was used to separate pigment-protein complexes in ICM samples prepared from microaerophilically grown cells.

Tracks: (1) 'Tn₅I'; (2) wild type RB4; (3) Km-sensitive revertant; (4) Km-resistant revertant.

Acrylamide concentration 7.5% (w/v); Loading 150 μ g protein/track; Gel was photographed after silver staining.

resistant to Km and Sm whilst the rest were sensitive to both of these antibiotics. These two classes were assumed to be due to transposition to another part of the genome and transposon-loss by precise excision, respectively.

One antibiotic-resistant and one sensitive revertant were picked, grown up in liquid culture, and spectra taken of cell free extracts. Both exhibited a normal wild-type spectrum. Figure 7.6 shows the semi-denaturing PAGE of membranes prepared from the revertants compared to membranes prepared from the mutant 'Tn5' and the original wild-type. It shows clearly that both the pigment-protein complexes (LHI and LHII) which were absent in the mutant 'Tn5I' have been restored by reversion. Both revertants exhibited the same protein profile as the wild-type. Thus this 'light lethal' selection proved to be a very powerful method for selection of precise excision events. More important, it provided a genetic proof that the 'Tn5I' mutant phenotype was indeed attributable to a single Tn5 insertion.

7.7 Analysis of Tn5 insertion mutants and revertants by Southern hybridization

Problems encountered in transposon mutagenesis include the possibility of insertional specificity and multiple insertions. Insertional specificity of Tn5 has been reported in Acinetobacter calcoaceticus, where it was shown to have a single site of insertion in the chromosome (Singer and Finnerty, 1984). Therefore it is desirable to demonstrate the physical presence of the transposon in one site for a particular mutant, ie to show that the mutant phenotype is due to one insertion only. It is also desirable to show that the site of insertion is random

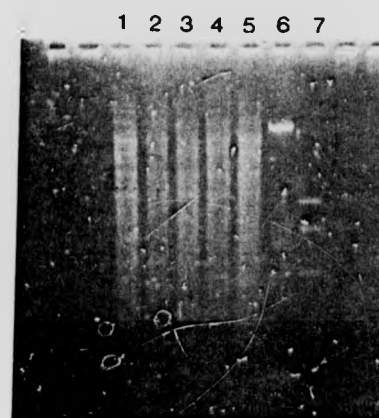


Figure 7.7a ClaI digests, of genomic DNA purified from Tn5-
insertion pigment mutants and 'Tn5' revertants,
separated on an agarose gel.
(Tracks : as indicated opposite).

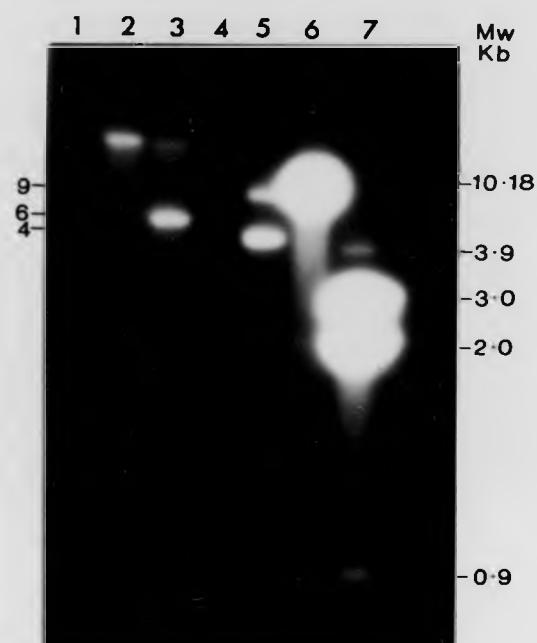


Figure 7.7 b Hybridization of 32 P-labelled HpaI internal fragment of Tn5 to ClaI digests of genomic DNA purified from Tn5-insertion pigment mutants and 'Tn5I'-revertants

Tracks: (1) wild type RB4, (2) 'Tn5I' green mutant; (3) Km^r -revertant of 'Tn5I' (4) Km^s -revertant of 'Tn5I'; (5) 'Tn5II' orange/brown mutant; (6) pBR::Tn5 digested with EcoRI (7) pBR::Tn5 digested with BamHI/XmaI-M.wt. markers. <photo.-contact print>

for different mutants. This can be achieved by DNA-DNA hybridization using a length of DNA specific to Tn5 to probe restriction digests of DNA from various mutants.

To determine the status of Tn5 in the pigment mutants and the revertants, Southern blot analysis with a ³²P-labelled HpaI internal fragment from Tn5 was carried out. The restriction endonuclease ClaI was used to digest chromosomal DNA because Tn5 lacks its recognition site thus, on hybridization, single fragments of different sizes should be visualized for different insertions. The autoradiograph (Figure 7.7) shows that the internal fragment of Tn5 did not hybridize with wild-type DNA and did hybridize with both of the mutants 'Tn5I' and 'Tn5II'. In 'Tn5I' the expected single band was observed. 'Tn5II' had two bands and these were of different sizes to those in 'Tn5I' indicating different sites of insertion. The second band may be due to a secondary site of insertion of Tn5 or may be a band due to partial digestion of the DNA. The Tn5 fragment did not hybridize with the DNA from the Kanamycin-sensitive revertant of mutant 'Tn5I' and did hybridize with the Kanamycin-resistant revertant DNA. Again there were two bands, one of which might be due to a partially digested fragment or alternatively a secondary site of insertion of Tn5. These fragments were of different sizes to the bands that hybridized in the pigment mutants.

7.8 Discussion of analysis of Tn5-induced pigment mutants

Analysis of the pigment mutants isolated after transposon mutagenesis confirmed that they were transposon-induced. The green mutant 'Tn5I' was completely deficient in its photosynthetic apparatus, and reversion analysis confirmed that this phenotype was linked to the antibiotic

resistances carried by Tn5. The spectrum of this mutant suggested that the transposon was inserted in one of the genes for the synthesis of Bchl-a, resulting in the accumulation of a precursor with an absorption peak at 667nm. As discussed in Chapter 6, mature Bchl is a prerequisite for the assembly of pigment-protein complexes and their inclusion in the photosynthetic membrane of Rb. sphaeroides and Rb. capsulatus. Thus it is plausible that a transposon inserted in a gene required for Bchl synthesis could prevent assembly of the complete photosynthetic apparatus of Rm. vannielii as observed in mutant 'Tn5I'. Loss of the transposon by precise excision or transposition to another locus, as demonstrated by Southern hybridization, was accompanied by the complete restoration of photosynthetic capability. This provided genetic proof that the 'Tn5I' phenotype was due to a single Tn5 insertion.

7.9 Isolation of motility mutants by transposon mutagenesis using S17-1 (pSUP2021) (MacDonald, 1987)

Subsequent to the work described here, MacDonald (1987) used the S17-1 (pSUP2021) system to carry out transposon mutagenesis and isolate motility mutants of Rm. vannielii RB2. Km-resistant transconjugants were obtained at 1×10^{-7} and 1.2% of these were defective in motility as shown by the sloppy agar assay. 14% of the transconjugants were Cm-resistant and all of the non-swarming transconjugants were Km^r, Sm^r and Cm^s. These results, like those in this study, suggest that plasmid integration occurs as a secondary event to transposon insertion when using pSUP2021 for transposon mutagenesis in Rm. vannielii.

7.10 Conclusion

This work demonstrates that the technology of transposon mutagenesis works in Rm. vannieli. Although both pJB4JI and pSUP2021 show signs of plasmid integration, such events can be detected in screening. Also the integration of plasmids could be exploited in the development of a technique for chromosome mobilization. Transposon mutagenesis has already yielded pigment and motility mutants of Rm. vannieli and it is theoretically possible to isolate any mutant of choice (except essential genes), given an appropriate screening or selection procedure. Transposon Tn5 inserts non-specifically into the genome of Rm. vannieli as shown by reversion analysis and Southern hybridization.

However there may be some peculiarity in the transposition of Tn5 in this organism. The hybridization data showed that the transposon might possibly be inserted into one or more secondary sites in the chromosome (Figures 7.3 and 7.7). Future work should include checks for such events using hybridization techniques.

In addition there are potential problems of complementation in a RecA organism and the isolation of RecA mutants of Rm. vannieli should be a priority if further genetic work is to be carried out.

The transfer of Tn5 to Rm. vannieli indicates that there is no barrier to transfer of plasmids by conjugation to this organism. In the absence of a transducing phage, a plasmid which could replicate in Rm. vannieli is an essential tool in a system of genetic analysis. The next chapter describes the search for such a plasmid amongst a collection of broad host range plasmids and the cloning of the Tn5-containing fragment from 'Tn5I'.

CHAPTER 8

CLONING OF TN₅-CONTAINING FRAGMENT FROM 'TN₅I' AND
 TRANSFER OF BROAD HOST RANGE PLASMIDS TO RM. VANNIELII

8.1 Introduction

Having demonstrated that the phenotype of 'Tn5I' was due to a single insertion of the transposon, it was of interest to clone the 'tagged' gene. There were two possible approaches: to clone the transposon plus flanking regions into a standard E. coli vector (eg pBR322) where the DNA could be analysed in detail and manipulated with ease, or to clone into a broad host-range plasmid and transfer the plasmid back to 'Tn5I'. Use of the broad host-range system would allow selection for precise excision of the transposon by the positive selection method (Section 7.6). Because of the copy number effect of the plasmid, precise excision from a plasmid will occur at higher frequency than from the chromosome (Hinton et al., 1987). This would result in the original wild-type sequence being located on the plasmid. This chapter describes the former approach; ie cloning into pBR322 and the screening of a range of broad host-range plasmids for transfer to and maintenance in Rm. vanniellii.

8.2 Cloning of the EcoRI transposon carrying fragment of 'Tn5I'

Certain restriction enzymes do not have a recognition site in Tn5. By using such an enzyme and inserting fragments into a suitable vector it is possible to select plasmids carrying Tn5 plus the flanking sequences by virtue of the antibiotic-resistance carried by the plasmid. EcoRI was chosen here since the size of the EcoRI fragment had previously been determined. The EcoRI fragment that contained Tn5 was approximately 10kb (Section 2.28). 'Tn5I' chromosomal DNA was digested to completion with EcoRI (Section 2.25). Fragments were separated by electrophoresis on a 0.6% (w/v) agarose gel (Section 2.28) and DNA fragments in the size range 7.5 to 15kb were isolated (Section 2.31). This procedure enriched

for the Tn5-carrying fragment. Vector pBR322 was digested to completion with EcoRI in the presence of alkaline phosphatase to prevent self-ligation. 1 μ g of EcoRI-cut and phosphatased pBR322 DNA was ligated with 6 μ g of the EcoRI-cut chromosomal DNA (Section 2.26). Aliquots (10 μ l) of the ligation mix were added to competent HB101 cells. Following transformation (Section 2.34), cells were pooled and incubated in 10ml SOB (90 min, 37°C) to allow gene expression. Cells were concentrated by centrifugation and resuspended in 1ml SOB. Aliquots (100 μ l) were plated on NA plates containing Km at 20 μ g ml⁻¹ to select Tn5-containing cells. Km-resistant colonies (22) were isolated which fell into two classes on screening on Km, Ap and Tc. All were resistant to Tc and Km but only 5 were also Ap-resistant. 5 of each class were chosen for further analysis. Plasmids were prepared from each of the 10 clones (Section 2.24.2). Comparison of uncut plasmids with pBR322 by electrophoresis suggested that they were all larger than the parental plasmid (ie contained inserts) and that the plasmids from Ap-resistant clones were larger than the plasmids from Ap-sensitive clones. This suggested that in the Ap-sensitive clones the Ap-resistance gene had been deleted. Digestion with EcoRI confirmed that some alteration had occurred in the 'Ap-sensitive' plasmids, as only one fragment of about 12kb was obtained (Section 2.28) instead of the two expected from the insertion of an EcoRI fragment into an EcoRI site. Digestion of the 'Ap-resistant' plasmids with EcoRI (Figure 8.2) confirmed the insertion of an EcoRI fragment of about 9.2kb in 4 clones. The fifth contained two fragments (7.5 and 10kb). This preliminary analysis suggests that 4 of the Ap-resistant clones carried plasmid with a single Tn5-containing insert (further restriction mapping is required to confirm this) and the fifth contained two fragments. It is surprising that the latter did not contain the 9.2kb fragment found in the other four. The presence of the

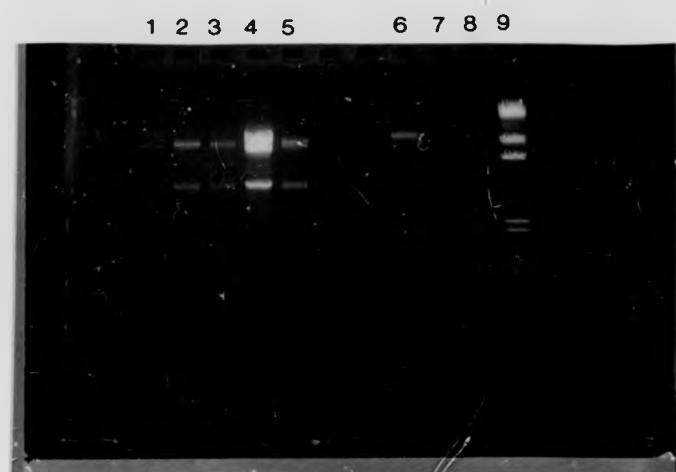


Figure 8.2 EcoRI digestion of plasmids prepared from clones

Tracks : 1 - 5 Ap-resistant clones

6 - 8 Ap-sensitive clones

9 λ -Hind III markers

Band sizes	1	2	3	4	5	6	7	8
	12931					11810	11810	11810
	9277	9277	9277	10011	9277			
	4098	4098	4098	7533				
				4098	4098			

two fragments in the fifth clone could result from the cloning of a partial EcoRI fragment or the attachment of non-contiguous chromosomal fragments during ligation.

8.3 Transfer of broad host-range plasmids to *Rm. vannielii*

8.3.1 Introduction

With the development of systems for carrying out mutagenesis and cloning of genes in any organism it becomes necessary to have a means of moving genes between strains for genetic mapping, strain construction and transfer from *E. coli* to the organism under study. Many species of bacteria have indigenous plasmids and phage, which in principle could serve as a basis for the construction of cloning vectors for the host organism. Some preliminary attempts to find such vectors for *Rm. vannielii* were described earlier (Chapter 3). However, considerable time and effort is required to develop a useful vector from a basic replicon and although a range of indigenous vectors have been found amongst the Rhodospirillaceae, capsduction using the gene transfer agent of *Rb. capsulatus* is the only example of the successful exploitation of an indigenous sytem.

Fortunately, in the case of Gram-negative bacteria, a number of plasmids exist which have the ability to replicate in a wide range of species. These are known as broad host-range plasmids and several have now been developed into cloning vectors which are useful for genomic cloning experiments in many Gram-negative bacteria (Franklin, 1985). The use of such plasmids has been the most successful approach to genetic analysis of the purple non-sulphur bacteria (Scholnik and Marrs, 1987).

The only successful attempt to transfer broad host-range plasmids to Rm. vanniellii was that by Potts (1980). On the basis of stimulation of frequency of Ap-resistance, she suggested that the IncP plasmid R68.45 had been transferred. Resistance to Tc and Km, the other markers on the plasmid, was not observed. Back-transfer experiments suggested that the complete plasmid had been maintained in Rm. vanniellii since all the markers were transferred to E. coli. Physical presence of the plasmid in Rm. vanniellii or visualization of the intact plasmid following transfer to E. coli was not demonstrated.

8.3.2 Initial matings with selection of transconjugants using gas-bag incubation and tube technique

Some preliminary experiments were carried out to find the optimal mating conditions. At that time the 'gas-bag' technique was still in use and no transconjugants were observed. A standard mating procedure (Section 2.16) was adopted based on the work of Potts (1980) and general mating techniques for broad host-range plasmids (Franklin, 1985). Experiments using RP4 with selection for transconjugants in tubes yielded some Km-resistant transconjugants. However it was found that the Ap and Tc markers were of little use since use of both these antibiotics (50 and $10\mu\text{g ml}^{-1}$ respectively) resulted in confluent growth (the former perhaps due to carryover of β -lactamase produced by E. coli from the mating and the latter due to the rapid degradation of Tc by the light). No conclusions could be made about the transfer of RP4 to Rm. vanniellii because the Km-resistant colonies could be spontaneous mutants. However the concurrent work with the suicide vectors pJB4JI (Chapter 7) suggested that plasmid transfer was occurring under the conditions used.

8.3.3 Transfer of broad host-range plasmids to *Rm. vannielii* using microaerophilic growth conditions to select transconjugants

During the course of this work a selection of plasmids from the IncP, Q and W incompatibility groups and suitable mobilizing vectors were obtained (Table 2.2). Plasmid markers were checked and plasmids prepared as described in Section 2.24.2. E. coli HB101 was transformed with the plasmids so that all were in an isogenic background. The plasmids used were non-conjugative but mob⁺ (ie can be co-transferred by a conjugative plasmid). Tri-partite matings were performed using pRK2013 to mobilize the other plasmids into Rm. vannielii. pRK2013 acts as a helper plasmid providing the necessary transfer (tra) genes required for mobilization. Matings were carried out as described in Section 2.16 and transconjugants were selected under microaerophilic conditions (Section 2.7). Tri-partite matings were carried out between E. coli strains to ensure that each plasmid was being mobilized.

Colonies of Rm. vannielii for viable counts appeared at about 10 days but to select transconjugants on antibiotics the plates had to be incubated for more than 14 days. Colonies came up very slowly on Tc at $10\mu\text{g ml}^{-1}$. Table 8.3.3a shows the results of the matings.

Transconjugants were isolated at frequencies greater than 8.5×10^{-7} per recipient and the highest spontaneous-resistance frequency for any antibiotic was 1.5×10^{-9} . The IncP plasmids pLAFRI and pGS72 showed the highest transfer frequencies (9.5×10^{-3} and 4.3×10^{-5} per recipient respectively). There was a great difference between the frequency of resistance to Km and Tc when using pGS72. This suggested that the concentration of Tc used for transconjugant selection was too high. The IncQ plasmids all had very similar conjugation frequencies (10^{-7}). The

Plasmid	Incompatibility Group	Antibiotic resistance markers	Selection antibiotic μl^{-1}	Frequency (per recipient)
pRK2501	P	Km, Tc	Km 25	5×10^{-7}
pLAFRI	P	Tc	Tc 10	9.5×10^{-3}
pGS72	P	Km, Tc	Km 25 Tc 10	4.3×10^{-5} 1.9×10^{-7}
pKT231	Q	Km, Sm	Km 25	6.4×10^{-7}
pKT240	Q	Km, Ap	Km 25	8.5×10^{-7}
pMMB33	Q	Km	Km 25	6.5×10^{-7}
pSF6	W	Sm, Sp	Sp	4.4×10^{-7}
pSa151	W	Km, Sm, Sp, Gm	Km Sp	3.05×10^{-6} 7.5×10^{-7}

Table 8.3.3a Transfer of broad host range plasmids to Rm.vannielii RB4

Plasmids were mobilized from E.coli HB101 by pRK2013 in tri-partite matings. Transconjugants were selected on PYM agar, with appropriate antibiotics, under microaerophilic growth conditions.

Plasmid	Original selection (antibiotic $\mu\text{g ml}^{-1}$)	Screening on selected and non-selected markers (% No. of patches that regrow/ No. of colonies screened)										Comments
		Km25	Sm25	Sm10	Ap50	Ap10	Tc10	Tc5	Gm5	Sp50		
pRK2501	Km 25	100					0	100				
pLAFRI	Tc 10						100*	nt				
pGS72	Km 25	100					88	nt			2 colony types: 12% larger-Tc ^s	
	Tc 10	100					100*	nt				
pKT231	Km 25	100	0	100								
pKT240	Km 25	100			0	100						
pMMB33	Km 25	100										
pSF6	Sp 50		0	100						100		
pSa151	Km 25	100	0	100					100	100		
	Sp 50	94	0	nt					nt	100	2 colony types: 6% larger Km ^s	

Table 8.3.3b Screening of transconjugants on selected and non-selected markers

Colonies were initially patched onto PYM plates containing antibiotics at the same concentrations as used for *E. coli* and later lower concentrations were used. Plates were incubated under microaerophilic growth conditions.

* - slow growing; nt - not tested

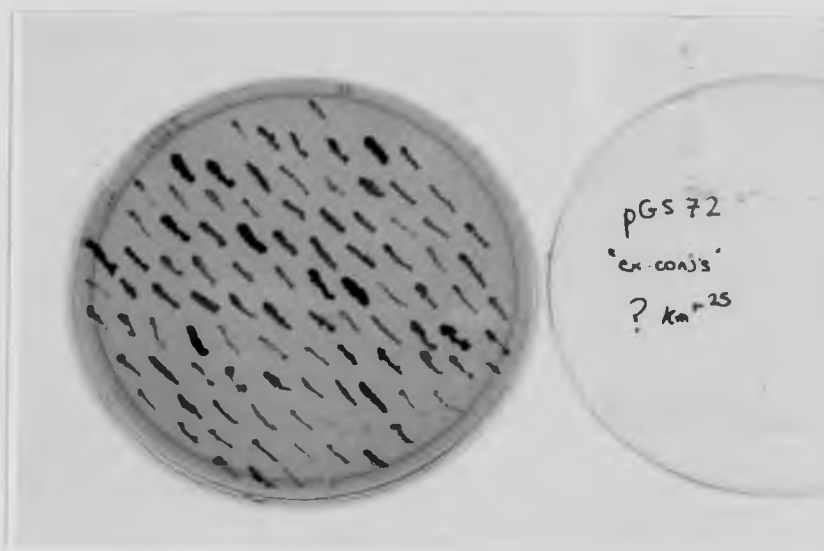


Figure 8.3.3 Two colony types obtained after transfer of pGS72 to
Rm. vannielii RB4

12% of Km-resistant colonies were larger and on screening on were found to be Tc-sensitive.

IncW plasmids had similar frequencies.

Table 8.3.3b shows the results of screening the transconjugants for selected and non-selected markers. There was 100% recovery on repatching on the selected markers in all cases. However when repatching on Tc it was observed that the growth was much slower than growth on other antibiotics. This correlated with the observation that transconjugants on Tc took a long time to appear. This suggested that growth was impaired at this concentration of the antibiotic and that perhaps lower concentrations should be used. Initially the transconjugants had been screened on antibiotics at the same concentration as used for E. coli. A different pattern was observed when they were re-screened on Sm, Ap and Tc at lower concentrations. The screening on non-selected markers suggests that all the plasmids with multiple resistances were transferred. pMMB33 and pLAFRI carried only one antibiotic-resistant gene so it was not possible to gain any information about their transfer by screening for a non-selected marker. However, the fact that the frequency of transconjugants was in the same range as that for the other plasmids, suggested that these two plasmids were also transferred. The observation of two colony types after matings with pGS72 and pSal51 was further evidence for plasmid transfer. The larger colonies were only resistant to the initial selective antibiotic, suggesting that they were spontaneous mutants. However, the weaker colonies were resistant to non-selected markers, suggesting that they carried the plasmid. An example of this heterogeneity is shown in Figure 8.3.3.

8.3.4 Discussion

The results suggest strongly that plasmids from all three

incompatibility groups, give resistance to a range of antibiotics and some of them have been developed for specific purposes, eg pKT240 is a promoter probe plasmid and pMMB33 is a cosmid. Thus there is the potential for numerous novel genetic manipulations in this organism. An interesting experiment would be to introduce the EcoRI fragment cloned from 'Tn5I' to pLAFRI or pSal51, transfer it back to the mutant and select precise excision of the transposon from the cloned sequence by positive ('light-lethal') selection. This assumes that the entire gene was present on the chromosomal DNA flanking the Tn5 element in the clone. As discussed above, precautions should be taken in such work to ensure that the transposon has inserted into a single site on the chromosome. Future work would be simplified by the isolation of RecA mutants of Rm.vannielii.

CHAPTER 9

GENERAL CONCLUSION

Prior to this project there were no techniques available for the genetic manipulation of Rm. vannieli. It has been shown, here, that the phototrophic incubation technique using the 'gas-bag' is not suitable for genetical experiments in this organism. In contrast, use of microaerophilic growth conditions, after mutagenesis of or transfer of plasmids to Rm. vannieli, yielded positive results. The reasons for this are not understood but it is suspected that the variability in temperature and dehydration of the agar restricted growth. Such factors would be more critical with the reduced growth rate in the presence of antibiotics. The microaerophilic growth technique gives controllable, reproducible conditions which are essential for genetic experiments. The physiology of this growth mode was not investigated here, but it merits further work. This work has demonstrated that there should be no obstacles to the isolation of any mutant that is required, given appropriate screening or selection techniques. It should now be possible to isolate developmentally-defective mutants including conditional lethal lesions in essential genes.

The technology of transposon mutagenesis was also found to work and the isolation of pigment mutants and motility mutants has been described. It is thus feasible to mutate and 'tag' any gene (except essential genes) in the Rm. vannieli genome with Tn5. Such transposon-tagged genes are easily cloned as described using pigment mutant 'Tn5I'.

The conjugation and screening experiments showed that broad-host-range plasmids of the Inc. P, Q and W groups can be transferred to Rm. vannieli. Further work is required to support these observations including isolation of plasmids from transconjugants and transfer of plasmids back to E. coli.

In conclusion the requirements of a basic system for genetic analysis have been fulfilled: genes can be mutated, cloned into E. coli for analysis or modification and returned to the original organism using an appropriate vector. The techniques described here should considerably enhance the potential of future workers to analyse the molecular biology of Rm. vannieli at the level of gene expression.

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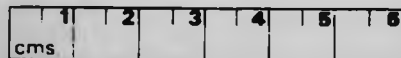
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